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The Development of Affinity Maturation in Rainbow Trout Oncorhynchus mykiss

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THE DEVELOPMENT OF AFFINITY MATURATION IN RAINBOW TROUT *ONCORHYNCHUS MYKISS*

A Thesis

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of

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DEDICATION

I would like to dedicate this thesis to my family, Dad, Mum, Liang Ing and Wei Tse, who have always supported me wholeheartedly in everything that I have done. Their love and faith in me have been a constant source of strength. Also, to my very good friends Becky, Evelyn, Jamie, Alessandra, Aswini, Renee, Tong, Aaron, Ai Ning and Jason I owe a debt of gratitude for their friendship and encouragement throughout this project.

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ABSTRACT

The purpose of this study was to characterize the process of affinity maturation in rainbow trout (*Oncorhynchus mykiss*) by both *in vivo* and *in vitro* methods, as well as the effect of aflatoxin B_1 (AFB_1), a fungal toxin, on the development of affinity maturation. Normal rainbow trout and rainbow trout that had been embryologically exposed to AFB_1 were immunized with trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) and given a secondary immunization 21 weeks later. Blood samples were taken prior to immunization (week 0) and at weeks 5, 12, 21 and 25 post-immunization in order to examine the change in affinity distribution of antibodies produced over the primary and secondary immune responses. The antibody titers and average affinities of the normal and AFB_1 fish were not significantly different and the groups were pooled to increase the accuracy of the affinity and titer measurements. The antibody affinity distribution at each time point was determined by a solid-phase affinity ELISA which partitions antibodies into subpopulations, each with a distinct affinity. There was a significant skewing of the *in vivo* affinity distribution from predominantly low affinity antibodies to predominantly high affinity antibodies between week 5 and week 12. This shift towards higher affinity antibodies was paralleled in the *in vitro* studies, suggesting an antigen-driven selection mechanism involving preferential stimulation of higher affinity antibodies by small limiting amounts of antigen. From the results of this project it is suggested that antigen becomes limiting between week 5 and week 12 and leads to the increase in affinity by antigen-driven selection in this timeframe.

PROJECT SUMMARY

Affinity maturation, or the temporal increase in the affinity of serum antibodies to their inducing antigen, is a well-documented feature of the mammalian specific immune response. In comparison, there has been limited work conducted on the temporal variations in affinity of specific antibodies and the underlying mechanisms in teleosts. Available data suggests that there is little or no affinity maturation in fish species (Fiebig et al., 1977; Lobb, 1985). However, these studies have largely employed techniques such as equilibrium dialysis and fluorescence quenching which can be used to determine the average intrinsic affinity of an antibody mixture, but do not allow for the partitioning and analysis of a complex mixture of antibodies. Using a more sophisticated solid phase, immunosorbent assay (Kaattari and Shapiro, 1994), complex mixtures of rainbow trout antibodies can be resolved into separate subpopulations with distinct affinities. Using this method, shifts in affinity distributions over time could easily be dissected and elements of the underlying mechanism revealed.

This project characterized the process of affinity maturation in rainbow trout (*Oncorhynchus mykiss*) over time using both *in vivo* and *in vitro* methods. Trout were immunized and blood samples taken at weeks 0, 5, 12, 21 and 25 . *In vitro* stimulations of peripheral blood lymphocytes from the blood samples at these time points were used to evaluate the cellular mechanism of *in vivo* affinity maturation as well as the capability of the cells to undergo antigen-driven selection of the high affinity B cells. Polyclonal activation of B cells was conducted using a non-specific mitogen in order to obtain an idea of the total number of B cells present. Cells were also stimulated with a high and a low dose of an antigen to determine how the various subpopulations of B cells changed with time and antigen dose. The high dose of antigen was used to stimulate an optimal number of specific B cells while the low dose was used to preferentially stimulate high affinity B cells. In this way the cellular mechanisms of affinity maturation could be determined.

THE DEVELOPMENT OF AFFINITY MATURATION IN RAINBOW TROUT *ONCORHYNCHUS MYKISS*

INTRODUCTION

Intrinsic and functional antibody affinity

The ability of an antigen to induce specific antibodies and the binding of these antibodies to the antigen are key elements in the protective immune response. Antibody binds antigen by means of reversible intermolecular interactions that include electrostatic forces, hydrogen bonding, hydrophobic forces and Van der Waals forces (Getzoff et al., 1988). The antibody binding site and epitope (amino acid residues of the antigen that comes into direct contact with antibody) possess complementary electron clouds which allow antibody and antigen to fit tightly together. The strength of binding of an antibody for its specific antigen, termed the antibody affinity, determines how effective the antibody will be in complexing with the antigen and facilitating its removal from the body. Consider the following equation:

$$Ab + H \Leftrightarrow AbH$$

where Ab is antibody and H is hapten (small monovalent antigen which binds to only one antibody binding site). If the antibody binds the hapten strongly, it is termed a high affinity antibody. The equilibrium constant, K, can be calculated by the following equation:

$$K = [AbH]/[Ab][H]$$

where [H] is the concentration of free hapten and [AbH] is the concentration of hapten bound by the antibody. Therefore,

$$\text{when } [AbH] = [Ab]$$

$$\text{then } K = 1/[H]$$

Thus, the equilibrium or affinity constant is defined as the reciprocal of the concentration of free hapten at the point where half of the antibody sites are bound (Getzoff et al., 1988). K in this case is considered a measure of the intrinsic affinity, that is, the affinity of one binding site of an antibody molecule for a specific antigen (Hornick and Karush, 1972).

However, immunoglobulin molecules are multivalent, possessing more than one antigen-binding site per molecule. The basic unit of any immunoglobulin molecule is the monomer, consisting of a pair of heavy chains and a pair of light chains. Each monomer, therefore, has at least two antigen-binding sites, with each binding site being composed of a heavy and a light chain. The immunoglobulin heavy and light chains each contain an amino-terminal variable (V) region which composes the binding site and differs between antibodies. The remaining carboxy-terminal regions of the heavy and light chains are invariable within an isotype and are termed the constant (C) regions. The specificity of an immunoglobulin molecule for a specific antigen is determined by the amino acid sequences of the variable regions of the heavy and light chains (Kaartinen et al., 1983). In mammals there are five major immunoglobulin (Ig) isotypes, IgG, IgA, IgM, IgD and IgE, which differ in their constant region genes (reviewed by Honjo et al., 1989). IgG and IgA can also be divided into subisotypes in some species. The largest Ig molecule is IgM which is a pentamer and consists of five subunits or monomers (Miller and Metzger, 1966). The IgM molecule thus possesses a total of ten binding sites. The IgG, IgD, IgE and some IgA molecules are monomeric and possesss a total of two antigen-binding sites. Since the majority of antigens possess multiple identical epitopes, the interactions between a single antigen and a single antibody can be complex. The strength of this bi- or multivalent interaction is referred to as the avidity or functional affinity of the antibody. The functional affinity is greater than the sum of the individual interactions between an antibody binding site and an individual epitope. The initial bonding of one epitope to a binding site facilitates and enhances the strength of additional interactions between that antibody and antigen (Hornick and Karush, 1972; Karush, 1976; Novotny et al., 1989).

Affinity maturation

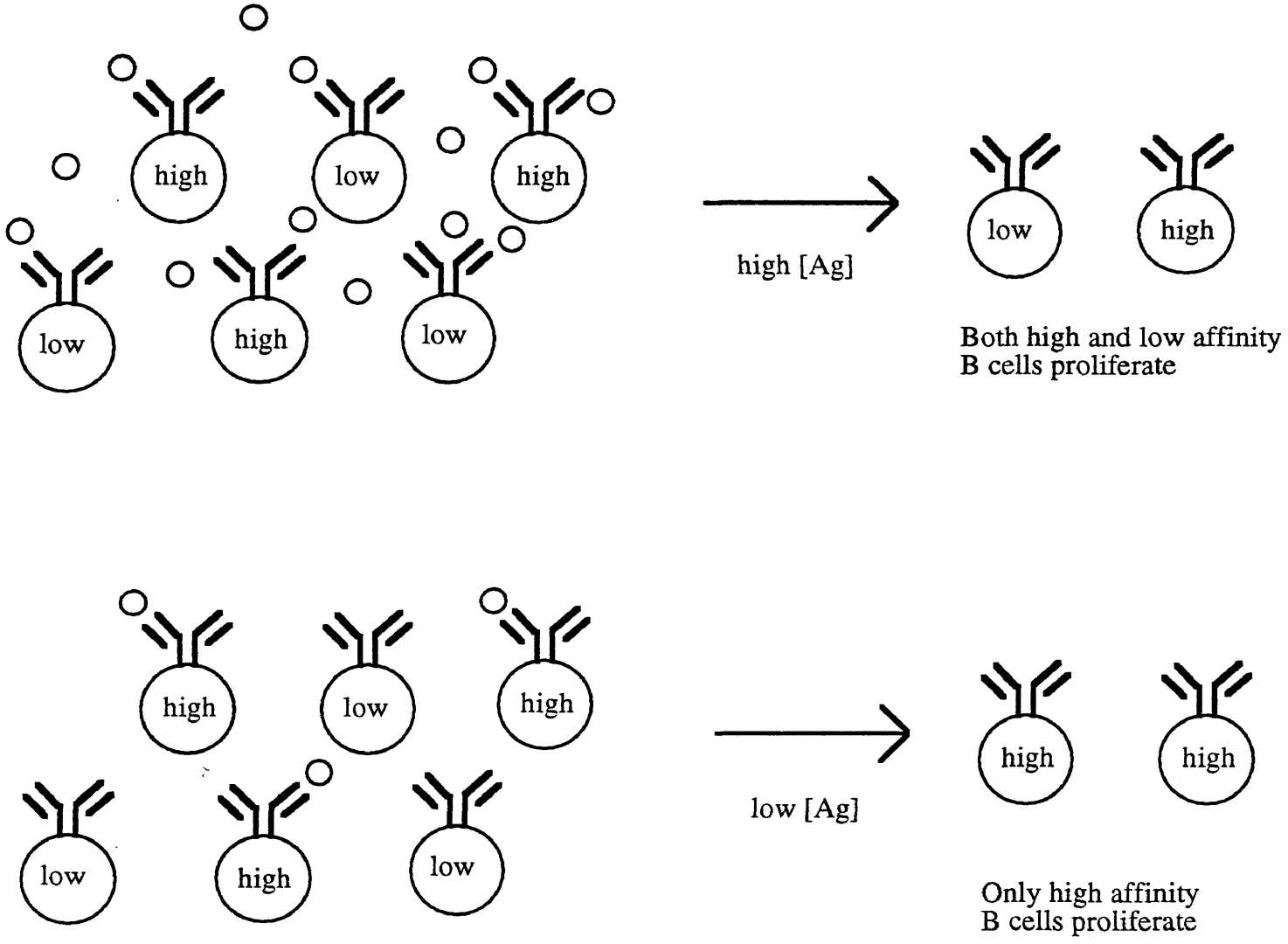
The affinity of serum or plasma antibody is not a static feature of the immune response. Affinity maturation, the increase in antibody affinity with time, is an essential and well-

documented immunological feature in mammals (Eisen and Siskind, 1964; Goidl et al., 1968). When a mammal is initially immunized with an antigen, the antibodies produced are mostly of the IgM isotype and are of relatively low affinity. In response to antigenic stimulation a series of events at the cellular and DNA level occurs that brings about a switch in the class of secreted antibody from IgM to IgG (Wabl et al., 1978; Andersson et al., 1978; Van der Loo et al., 1979; Honjo et al., 1989). The intrinsic affinity of the IgG antibody can eventually be 10 to 100 fold higher than that of IgM (Onoue et al., 1968). (However, IgM can still bind multivalent antigens effectively because of the beneficial effects of multiple antigen-antibody interactions, as previously explained). Thus, in mammals there is a switch in antibody class that is accompanied by an increase in intrinsic affinity of the secreted antibody.

In mammals there are two processes involved in affinity maturation; 1) antigen-driven selection of B cell clones (Eisen and Siskind, 1964; Siskind and Benacerraf, 1969; Goidl et al., 1969) and 2) somatic mutation within these clones (Griffiths et al., 1984; Berek et al., 1985; Maizels, 1995). According to the antigen-driven selection or clonal selection theory, when an antigen comes into contact with B cells, it preferentially stimulates the proliferation of these cells via interaction with antibody receptors that are complementary to the antigen (Burnet, 1959; Lederberg, 1959). After the introduction of antigen by immunization or infection, the antigen concentration slowly declines in the body. Initially, when the concentration of antigen molecules is highest, they can bind to and activate a large number of B cells possessing receptors of high or low relative affinity for the antigen (Figure 1). These activated B cells will proliferate, differentiate and produce antibodies identical to their receptors. Thus the early antiserum contains antibodies possessing a wide range of affinities. Over time the concentration of antigen in the animal decreases so that only progressively higher affinity B cells will be able to bind sufficient antigen to become activated and undergo clonal expansion. Thus, there is preferential expansion of B cell clones that produce antibody with high affinity for the antigen, leading

FIGURE 1. The selection of high affinity B cells by antigen. Initially after immunization, the high concentration of antigen stimulates both high and low affinity B cells to proliferate and produce antibodies of both high and low affinities. As time progresses the level of antigen declines so that later in the immune response only high affinity B cells can bind sufficient antigen to be stimulated to proliferate. This leads to an increase in the predominance of high affinity antibodies.

FIGURE 1. The theory of antigen-driven selection



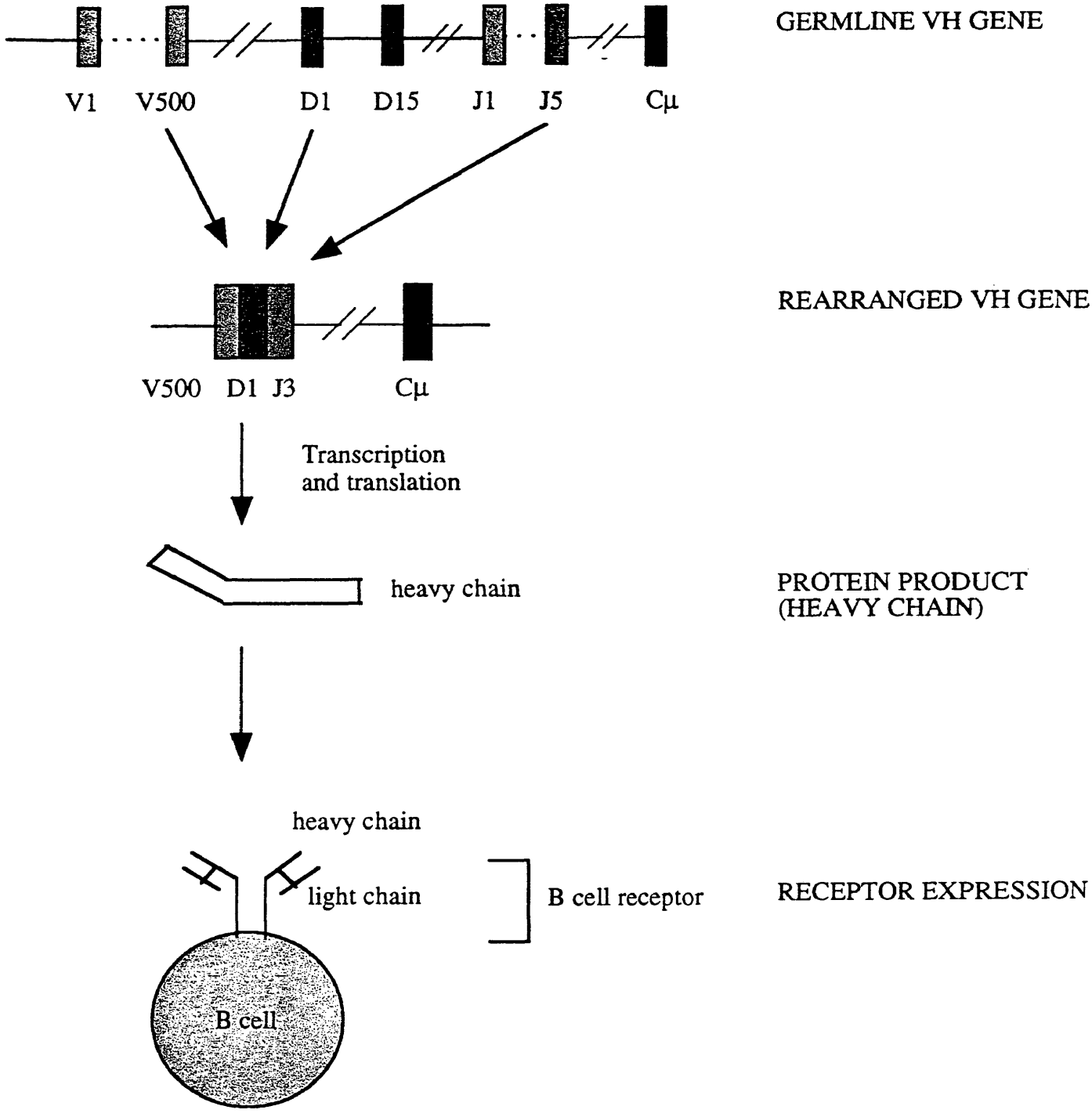
to the progressive increase in the average affinity of the expressed antibodies over time (Siskind and Benacerraf, 1969; Liu et al., 1989).

In mammals, recombination of germline gene elements is the initial event that provides a tremendous amount of antibody diversity to cope with the myriad number of antigens encountered by the immune system (reviewed by Tonegawa, 1983). In mammals the variable region of the immunoglobulin heavy chain is encoded by three gene segments, V_H, D_H and J_H, and the variable region of the light chain is encoded by two gene segments, V_L and J_L. There are multiple V_H, D_H and J_H gene segments and eight to nine types of constant region heavy-chain (C_H) genes, depending on the species. The C_H genes are designated $C\mu$, $C\partial$, $C\gamma 1\text{–}4$, $C\varepsilon$ and $C\alpha 1\text{–}2$ (reviewed by Honjo et al., 1989). The V_H, D_H and J_H segments recombine to form a VDJ unit that comprises the variable region of the immunoglobulin heavy chain (figure 2). This VDJ unit then associates with a constant (C_H) region gene to form the transcriptionally active Ig heavy chain gene. The different combinations in which the V_H, D_H and J_H gene segments can be rearranged to form the recombined heavy chain variable region sequence and the V_L and J_L segments to form the recombined light chain variable region sequence, as well as the different pairings of the completed heavy and light chains to form the final antibody molecule generate considerable diversity (Tonegawa, 1983; Griffiths et al., 1984). Two other diversifying processes occur in the joint regions between gene segments and are termed junctional site diversity and junctional insertion diversity. Junctional site diversity is generated at the V_H-D_H, D_H-J_H and V_L-J_L junctions due to imprecise joining of the 5' and 3' ends of these gene segments. Junctional insertion diversity is generated only in the V_H-D_H and D_H-J_H junctions due to the insertion of several nucleotides. Recombination of gene segments, junctional diversity and junctional insertion diversity provide the initial broad repertoire of antibody receptor molecules upon which the process of antigen-driven selection can act.

Somatic mutation, or nucleotide changes leading to amino acid substitutions throughout the variable region, is the fourth diversifying process (Tonegawa, 1983).

FIGURE 2. Assembly of an immunoglobulin (Ig) heavy chain gene and expression of the gene to produce the Ig molecule. Germline V_H, D_H and J_H gene segments rearrange to form the variable region gene of the heavy chain. This rearranged variable region gene then combines with a constant region gene (in this case, Cμ) to form the completed heavy chain gene sequence. Similarly, germline V_L and J_L genes rearrange to form the variable region of the light chain (not shown). The expressed heavy and light chains are then assembled by means of disulfide linkages between the chains to form the Ig molecule. For example, two heavy and two light chains are assembled to form an Ig monomer.

Assembly of the immunoglobulin heavy chain gene and its expression to form the immunoglobulin molecule.



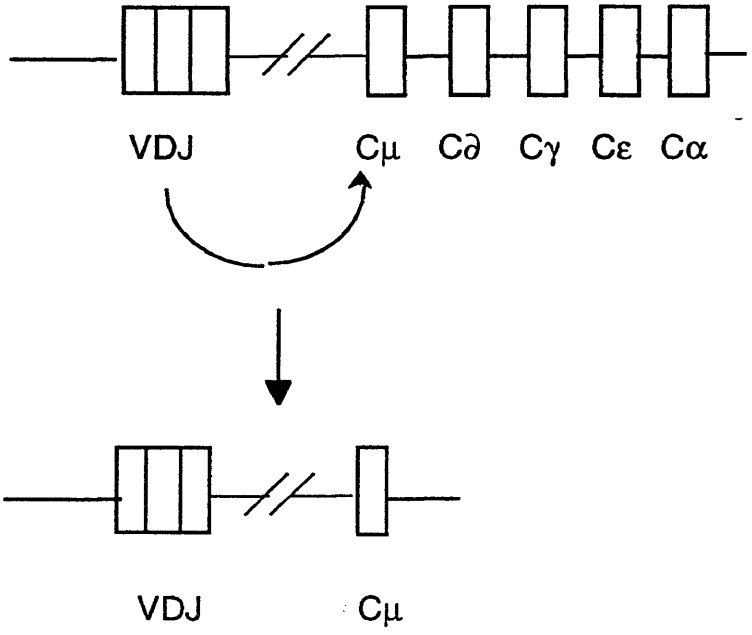
Antibody diversification via this process occurs after the previous recombinatorial processes have generated mature, transcriptionally-competent B lymphocytes bearing antibody receptors. Somatic mutation minimally requires that the B lymphocyte bind antigen, receive signals from a T helper cell (Neuberger and Milstein, 1995) and initiate transcription of the rearranged antibody genes (Storb, 1996). Thus, this process specifically acts to diversify antibody specificities during the immune response to the inducing antigen.

Somatic mutation can increase, decrease or have no effect on the affinity of the antibodies produced (Liu et al., 1989; Chen et al., 1995). Antigen-driven selection acting on these lymphocytes leads to preferential induction of the higher affinity somatic mutants as time progresses during the immune response. Griffiths et al. (1984) demonstrated that an increase in specific antibody affinity over time was accompanied by the occurrence of somatic point mutations in the antibody variable region gene, suggesting a role for somatic mutation in the affinity maturation process. Scott et al. (1994) reported that affinity maturation of autoantibodies was associated with a high replacement (mutations that actually result in amino acid replacement) to silent ratio of somatic mutations in the CDR (complementarity determining region) or antigen-binding region of the antibodies. Other investigators have also demonstrated an increase in affinity by artificially engineering point mutations in the antibody variable genes (Allen et al., 1988; Kocks and Rajewsky, 1988). This supports the idea that the increase in affinity of antibodies is accomplished by means of a concerted effort, with antigen selection and somatic mutation as the key players.

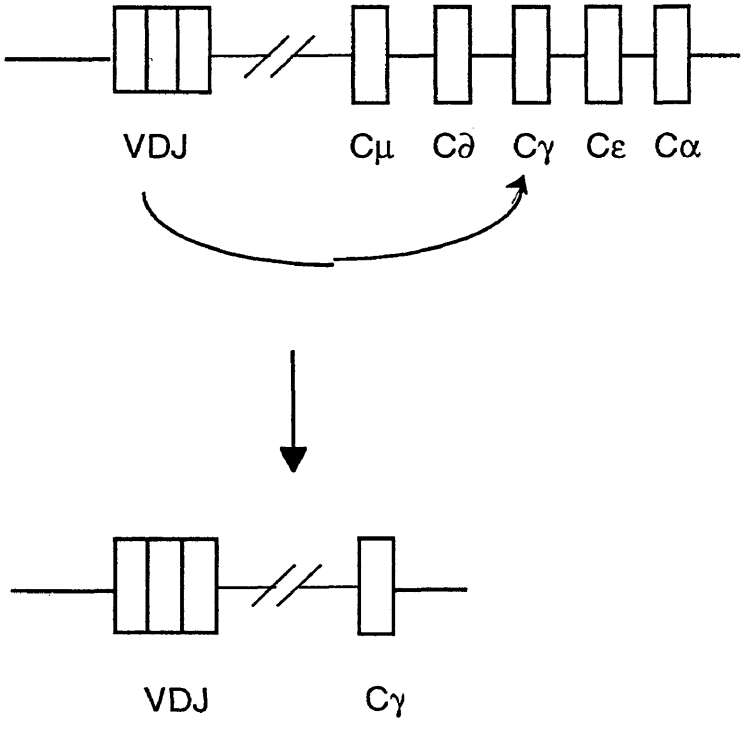
In mammals the process of affinity maturation has been associated with an additional process termed isotype switching. In response to antigen stimulation of a mature B cell, the recombined variable region gene ($V_HD_HJ_H$ unit) can "switch" and combine with another C_H gene (figure 3). This results in the expression of the same binding site in the context of another antibody class or isotype. During the specific immune response in mammals, a series of events occurs at the cellular and DNA level, causing cells to switch

FIGURE 3. The mechanism of isotype switching in mammals. Following antigen stimulation of a B cell, the heavy chain variable region gene (rearranged VDJ unit) can "switch" and bind to a different constant region gene, leading to the expression of a different antibody isotype. In this illustration, the variable region gene originally combined with a Cμ constant region gene. This completed gene sequence codes for the heavy chain of the IgM isotype. Following "switching", the variable region gene associates with a Cγ gene to code for the heavy chain of the IgG isotype. Thus there is a switch in the secreted antibody from IgM to IgG.

The mechanism of isotype switching in mammals.



FOLLOWING ANTIGEN STIMULATION OF B-CELL:



from the expression of IgM to IgG (Anderson et al., 1978; Gearhart et al., 1975; Van der Loo et al., 1979; Wabl et al., 1978). The IgG antibody has the same antigen specificity but eventually expresses a higher intrinsic affinity than IgM. This is explained by the fact that IgG antibodies contain a greater number of somatic mutations than their IgM counterparts with the same antigenic specificity (Gearhart et al., 1981). This differential expression of somatic mutants and higher affinities among IgG antibodies lends additional support for the theory that somatic mutation, in conjunction with antigen selection, can lead to the increase in antibody affinity.

Historically, affinity maturation was first characterized in the rabbit. Variations in the affinities of anti-dinitrophenyl (DNP) antibodies induced in rabbits were monitored over the duration of the immune response by Eisen and Siskind (1964). These investigators injected rabbits with DNP-bovine-gamma-globulin (DNP-BGG) and determined the antibody affinity by fluorescence quenching and equilibrium dialysis. The antibody affinity varied predictably during the immune response depending on the period of time that had elapsed after immunization and on the amount of antigen injected. The antibody affinities increased with time after immunization or increased when smaller amounts of antigen were injected. The potential for antigen-driven selection was also investigated by performing immunoprecipitation experiments with an anti-DNP antiserum. Successive additions of small amounts of inducing antigen to the antiserum resulted in the formation of precipitates which progressively included antibodies of decreasing affinity for the hapten (monovalent DNP). This supported the theory that when a limiting amount of antigen is added to a heterogeneous mixture of antibodies, antibodies of the highest affinity preferentially bind to the antigen (Eisen and Siskind, 1964).

Affinity maturation has been well characterized for IgG. Devey et al. (1990) demonstrated a marked affinity maturation of IgG in humans between weeks 1 and 3 following primary immunization and by week 1 following secondary and tertiary immunizations. Using affinity distribution plots, the authors could correlate this increase in

affinity with an emergence of high affinity antibody subpopulations between weeks 2 and 3 following primary immunization. These high affinity subpopulations persisted until a year post primary immunization. These researchers also discovered that when the subjects were immunized eight years later, there was a rapid shift from low affinity subpopulations before immunization to high affinity subpopulations post-immunization, corresponding to a likely clonal expansion of high affinity B cells upon immunization.

The temporal variations in distribution and heterogeneity of antibody affinity in rabbits were studied by Werblin et al. (1973). They reported that in the initial immune response, there is a symmetrical distribution of low affinity antibodies. At a later time after immunization there is shift to a skewed distribution featuring predominantly high affinity antibodies. The investigators found that the whole range of antibody subpopulations that is expressed by the rabbits over the course of the immune response is already observed by day 42 post-immunization. The change in average affinity of the antibodies is thus accounted for by a shift in the relative amounts of antibody subpopulations that were present early in the immune response (day 42) and not by the emergence of new antibody species. These findings provided further support for the theory of antigen selection.

IgM does not appear to undergo as dramatic an increase in affinity as does IgG. However, its multivalent binding of antigens enhances its functional affinity for multivalent antigens (Hornick and Karush, 1972). IgM antibodies secreted in the later part of the immune response can also display somatic mutations, indicating that class switching is not a requirement for somatic mutations (Griffiths et al., 1984). Thus, IgM has the potential to undergo affinity maturation, albeit most likely to a lesser extent than IgG.

Teleost antibodies and affinity maturation studies

The degree of affinity maturation exhibited by mammalian IgG has not been observed in lower vertebrates such as teleosts and elasmobranchs. Thus far only one class of immunoglobulin (a tetramer) has been reported in teleosts (Acton *et al.*, 1971, Voss et al.,

1978). These tetramers are composed of eight light chains and eight heavy chains possessing a molecular weight of approximately 700 kDa. Researchers have characterized some heterogeneity of these heavy and light chains in various teleost species, but the Ig molecules are always present as tetramers. Using an anti-trout Ig monoclonal antibody and polyacrylamide-gradient gel electrophoresis, Sanchez et al. (1989) elucidated two heavy chain forms and two light chain forms of rainbow trout Ig with different molecular weights and comprising different proportions of total Ig (Sanchez at al., 1989; Sanchez and Dominguez, 1991). Channel catfish (*Ictalurus punctatus*) produce at least three antigenically distinct heavy chains of tetrameric immunoglobulin, differentiable by monoclonal antibodies (Lobb and Olson, 1988) and at least two Ig heavy chains of different molecular weights and different relative abundances as determined by SDS-PAGE (Phillips and Ourth, 1986). Three light chains of channel catfish Ig with different molecular weights were elucidated by SDS-PAGE (Lobb and Clem, 1983). Lobb and Olson (1988) had referred to the heavy chain antibody forms as isotypes as they appeared to comprise consistently sized subpopulations with every individual catfish examined. However, there has been no evidence to date of unique and consistent differences between these so-called "isotypes", nor any genetic or sequence data that reveals the existence of specific C_H genes encoding these antigenically distinct forms.

The logarithmic increase in antibody affinity which is characteristic of the mammalian IgG response has not been observed in teleosts. Lobb (1985) observed a maximum two-fold increase in the intrinsic affinity of channel catfish anti-DNP antibodies. The intrinsic affinity constant (K) of the anti-DNP antibodies increased slightly from 5.6×10^6 M-1 at three months after primary immunization to 8.6×10^6 M-1 at seven months and then to a maximum of 11×10^6 M-1 at 15 months. Lobb interpreted these results to imply that catfish Ig contains relatively homogeneous antibody sites which show little increase in binding affinity with time post-immunization (Lobb, 1985).

Similarly, Killie et al. (1991) demonstrated a maximum two-fold increase in affinity of Atlantic salmon (*Salmo salar*) anti-NIP antibodies from a Ko value of 3.4×10^5 M-1 at two months post primary immunization to 8.4×10^5 M-1 at four months post primary immunization. Voss et al. (1978) compared the temporal change in affinity of coho salmon (*Oncorhynchus kisutch)* anti-fluorescyl antibodies with rabbit anti-fluorescyl IgG. From three weeks to eight weeks post primary immunization the average intrinsic affinity constant for the salmon antibodies increased from 4.3×10^5 M^{-1} to 4.7×10^5 M^{-1}. By contrast, from about two weeks to about nine weeks post primary immunization, the average instrinsic affinity of the rabbit IgG increased from 1.1×10^5 M^{-1} to 1.9×10^7 M^{-1}, an increase of more than a 100-fold. Although it appears that teleost antibodies do not undergo as dramatic an affinity maturation response as mammalian IgG, the literature suggests that teleost antibodies could experience an increase in affinity, albeit a proportionately smaller one, during the primary immune response. It has been proposed that the relatively low intrinsic affinity of salmonid antibody is compensated by the polyvalent (multiple antigen-binding sites) nature of the tetrameric Ig form, leading to a higher functional affinity or avidity (Voss et al., 1978). Fiebig et al. (1977) reported that intrinsic affinity values for carp (*Cyprinus carpio*) anti-DNP antibodies changed only slightly during the immune response whereas functional affinity values increased from 10^{10} M^{-1} late in the primary response to 10^{12} M^{-1} upon secondary immunization.

A number of investigations examining the ladyfish (*Elops saurus*) (Amemiya and Litman, 1990), catfish (Ghaffari and Lobb, 1991), goldfish (*Carassius auratus*) (Wilson et al., 1991) and rainbow trout (Roman and Charlemagne, 1994) have revealed that the heavy chain (IgH) gene locus in teleosts is organized in a similar manner to that of mammals. As in mammals, clusters of V genes precede D gene clusters which precede a J gene cluster. Multiple IgH gene families have also been elucidated in various teleost species. Nine IgH-V gene families have been characterized in rainbow trout (Roman and Charlemagne, 1994) and six in channel catfish, encompassing a total of 100 IgH-V genomic sequences

(Ghaffari and Lobb, 1991). This suggests that teleosts have the capacity to exhibit a comparable amount of antibody diversity as has been observed in mammals. The genetic platform is present with the potential to produce a repertoire of antibodies expressing a variety of affinities to a specific antigen. Additionally, Hinds-Frey et al. (1993) demonstrated junctional diversity and somatic mutation in the V_H repertoire of the primitive elasmobranch (*Heterodontus*) and suggested that somatic mutation evolved as a mechanism to contribute to antibody diversity prior to the evolution of combinatorial joining and extensive diversification of germline sequences. This is supported by the finding that many shark V, D and J segments are fused within the germline. This germline arrangement would preclude employment of somatic recombination, junctional diversification or junctional insertion in the generation of antibody diversity.

Although the above is true for elasmobranchs, the possibility that teleost fish resemble mammals in possessing mechanisms of somatic mutation and antigen-driven selection, leading to the preferential expression of high affinity antibodies with time has not been formally demonstrated. As yet no efforts have been reported that address the existence of somatic mutations in teleost fish, nor has the process of affinity maturation been examined with the express purpose of observing the development of high affinity variants during the immune response. Therefore the objectives of this project were to: 1) determine if affinity maturation occurs in rainbow trout, 2) determine whether new high affinity variants appear as the response matures and 3) determine if exposure to graded concentrations of antigen *in vitro* reveal higher affinity variants.

MATERIALS AND METHODS

Animals

Naive outbred Shasta strain rainbow trout, *Oncorhynchus mykiss,* ~ 2 years old, weighing 500 g - 1 kg, were tagged and kept in a UV irradiated, biofiltered pathogen-free recirculating system at 14°C. Fish were fed 4-6 g/fish daily of Trout Grower High Fat feed (Zeigler Brothers Inc., Gardners, PA).

Antigens and Mitogens

The antigen used in immunization was trinitrophenylated keyhole limpet hemocyanin (TNP-KLH). The hapten trinitrophenyl (TNP) was conjugated to a carrier protein keyhole limpet hemocyanin (KLH) that serves as an immunogen to stimulate the immune response. Lipopolysaccharide (LPS) from *E. coli* O111:B4 (Sigma, St. Louis, MO) was used as the mitogen to stimulate cell proliferation in the *in vitro* studies. A 10 mg/ml stock solution of LPS was prepared by dissolving 100 mg of the lyophilized powder in 10 ml RPMI-1640 and pasteurizing at 70°C for 30 min. Trinitrophenylated lipopolysaccharide (TNP-LPS) was prepared by conjugating LPS to TNP by the same procedure used in the preparation of TNP-KLH.

Immunizations and bleeds

Fish were initially immunized i.p. with 800 μg/kg of the antigen trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) in Freund's Complete Adjuvant (Sigma, St. Louis, MO). A secondary immunization of 400 μg/kg TNP-KLH in Freund's Incomplete Adjuvant (Sigma, St. Louis, MO) was administered at week 21. The fish were anesthetized in 10% benzocaine and bled before immunization (prebleed, week 0), during the early primary immune response (first bleed, week 5), during the late primary immune response (second

bleed, week 11), just prior to the secondary immunization (third bleed, week 20) and during the secondary immune response (fourth bleed, week 25). Approximately 1 ml of blood was extracted from the caudal vein using a 22 gauge, one-inch *Vacutainer* needle (Becton Dickinson, Franklin Lakes, NJ) and collected in tubes containing heparin, which acts as an anti-coagulant. The blood samples were centrifuged at 500 g in a Sorvall RT 6000D centrifuge (DuPont Equipment, Duluth, GA) to pellet the cells and the plasma was then decanted and saved for analysis.

Isolation and culture of peripheral blood lymphocytes (PBLs)

The pelleted cells obtained from the above procedure were resuspended in 10 ml RPMI-1640 (Sigma, St. Louis, MO) and centrifuged at 500 g for 10 min. to wash the cells. The supernatant was decanted and the cells were resuspended in 17 ml of fresh RPMI-1640 and mixed well. The cell mixture was carefully layered over 20 ml Histopaque 1077, which has a density of 1.077 ± 0.001 g/ml (Sigma, St. Louis), in a 50 ml centrifuge tube and centrifuged at 500 g for 45 min. The buffy coat layer (PBLs) between the RPMI and the Histopaque layers was removed with a pipet and the cells were then washed by centrifugation at 500 g for 5 min. The cell pellet was resuspended in 10 ml RPMI-1640 and centrifuged at 500 g for 10 min. to wash the cells. The RPMI-1640 was decanted and the cells were resuspended in 1 ml tissue culture media or TCM (Yui and Kaattari, 1987). The TCM is a modified Mishell and Dutton medium and consists of 20% fetal bovine serum in RPMI-1640, supplemented with 0.1% gentamicin sulfate, 0.9% 1mg/ml guanine, 0.9% 1mg/ml nucleosides (adenosine, cytosine, uracil) and 0.05% 50 µM ß-mercaptoethanol. The number of viable lymphocytes/ml TCM was determined by use of a hemacytometer and trypan blue exclusion . The cells were suspended in an appropriate volume of TCM to obtain a final concentration of 2×10^7 cells/ml, and 50 µl of the cell suspension was dispensed into each well of a 96-well cell culture plate. The cells were stimulated as follows:

1) 100 μg/ml LPS to provide polyclonal activation.

2) 1 μg/ml and 0.01 μg/ml doses of TNP-LPS to provide antigen-specific activation. The 1 μg/ml TNP-LPS was utilized as an optimal dose to stimulate all TNP-specific B cells while the 0.01 μg/ml TNP-LPS concentration acted as a low antigen dose to stimulate primarily high affinity TNP-specific B cells (Shapiro, 1996). The cultures were maintained at 17 °C in a blood gas atmosphere of 10% CO_2, 10% O_2 and 80% N_2 for 9 days. A stock supplement was prepared with 85% RPMI-1640, 9% RPMI-amino acids (Sigma, St. Louis, MO), 3% of a 20 mg/ml dextrose stock solution, 3% L-glutamine (Sigma, St. Louis, MO) and 0.06% of a gentamicin sulfate stock. Cells were fed every other day with a nutritional supplement consisting of 60% stock supplement, 30% fetal bovine serum, 4% 1 mg/ml nucleosides and 4% 1 mg/ml guanine. On the ninth day, the cultures were centrifuged at 500 g for 30 min and the supernatants were harvested. The supernatants were stored at -86°C in a Forma Scientific (Marietta, OH) freezer until analysis.

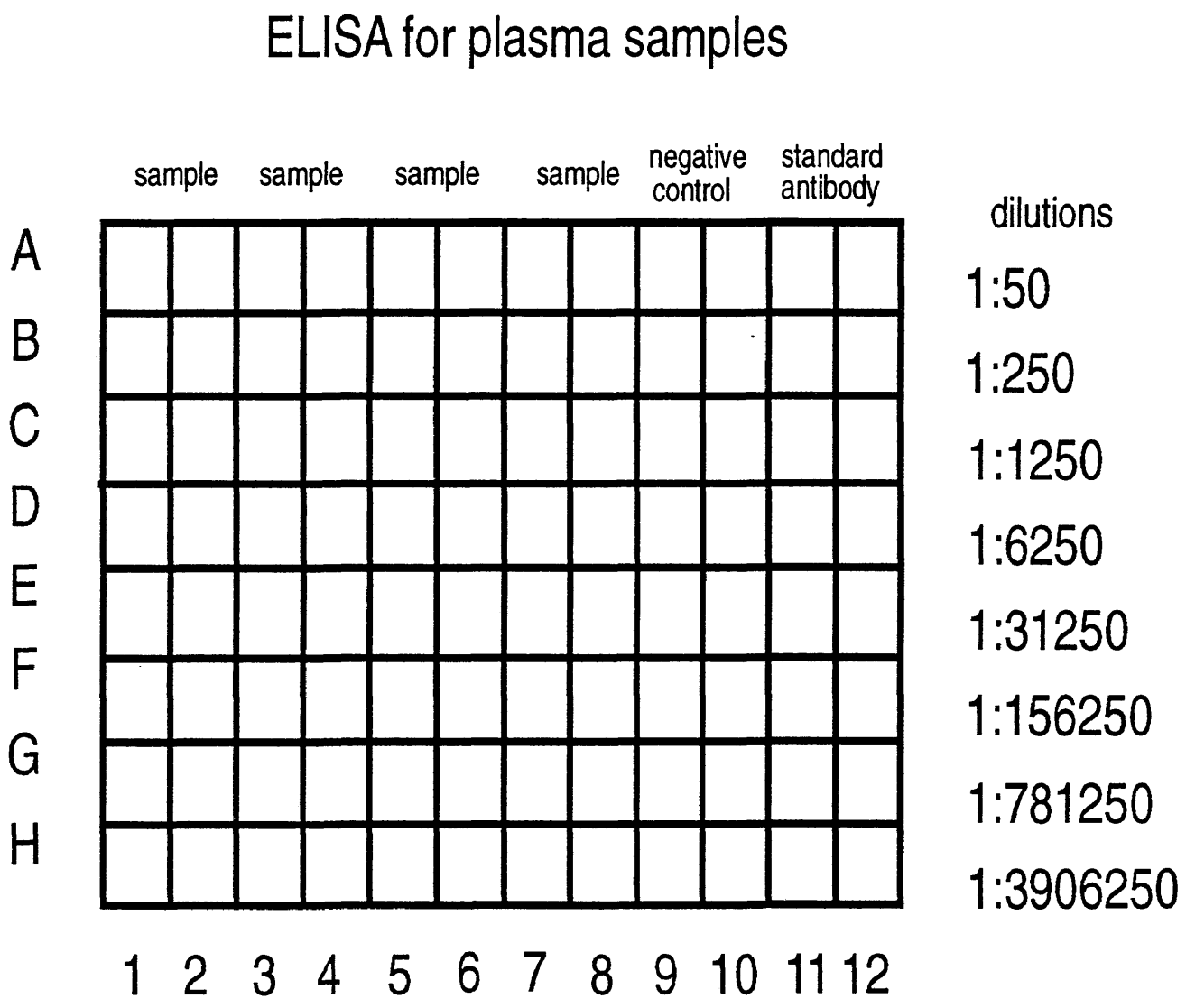
Determination of specific antibody titers

The amount of specific anti-trinitrophenyl (TNP) antibody in the plasma and culture supernatants was determined by an antigen-capture enzyme-linked immunosorbent assay (ELISA), adapted from Harlow and Lane (1988). The ELISA procedure for analyzing the plasma samples (Figure 4) involved coating the wells of a 96-well microtiter plate with 50 μl/well of 10 μg/ml trinitrophenylated bovine serum albumin (TNP-BSA) in coating buffer (0.02 M $NaCO_3$, 0.03 M $NaHCO_3$, pH 9.6) for one hour at room temperature. The plate was then washed three times with Tween 20 (1%) in Tris buffered saline (TTBS) using a *Titertek Plus* M96 plate washer (ICN Biomedicals, Inc., Cleveland, OH) and blocked with 240 μl/well of 1% bovine serum albumin (BSA) in TTBS for one hour at room temperature, after which the plates were washed three times with TTBS The plasma samples were diluted 1:50 in row A of a separate 96-well dilution plate followed by

sequential 1:5 dilutions in subsequent rows B-H. Each serially diluted plasma sample was loaded onto duplicate coated and blocked microtiter plate wells. A standard antibody of

FIGURE 4. ELISA plate design for assaying antibody concentration in plasma samples. Antigen is coated onto the wells of a 96-well microtiter plate. Following the blocking of non-specific binding to the wells, an initial 1:50 dilution is made of the plasma sample and placed in duplicate wells in row A. This is followed by serial 1:5 dilutions in rows B-H. A standard antibody of known concentration is diluted and placed in duplicate wells in a similar fashion. This generates a standard titration curve of O.D. versus plasma dilutions, from which the antibody titers of the samples can be determined. Buffer is placed in duplicate columns to serve as a negative control.

ELISA plate design for assaying antibody concentration in plasma samples.



known concentration (0.310 mg/ml protein) was serially diluted in a similar fashion and loaded onto the last two columns of the microtiter plate. The samples were then allowed to incubate overnight at 17°C. The plates were washed three times with TTBS and 100 µl/well of a 1:500 solution of biotinylated monoclonal mouse anti-trout antibody (1-14) was added to the wells and allowed to incubate for one hour at room temperature. The plates were washed three times with TTBS and then incubated with horseradish peroxidase-conjugated strepavidin (SA-HRPO) for one hour at room temperature. The SA-HRPO (Sigma, St. Louis, MO) was prepared according to the manufacturer's instructions. In the final step the plate was washed five times with TTBS and incubated with 100 µl/well of substrate solution for one hour. The substrate solution required for one plate consists of 10 µl of 30% hydrogen peroxide, 9.6 ml citrate buffer and 400 µl of 10 mg/ml 2,2 azino-bis(ethylbenzthiazoline-6-sulfonic acid) or ABTS (Sigma, St. Louis, MO) (Kaattari and Shapiro, 1994). The peroxidase and substrate react to give a colored product, the intensity of which (measured at 405 nm) is proportional to the amount of antibody that bound to the antigen (Figure 5). The optical density or O.D. was determined using a *Titertek Multiskan* MCC/340 microplate reader (ICN Biomedicals Inc., Cleveland, OH). The standard antibody was arbitrarily assigned a titer of 1000 units per µl and the titers of the plasma were determined by drawing a horizontal line across the titer curves at half of the maximum O.D. The volume of plasma which contained one "unit" of antibody activity (defined as the amount of antibody required to produce a color intensity of half the maximum O.D.) was determined from a plot of O.D. versus plasma volume (Figure 6). The ELISA procedure used to assay the culture supernatants was identical to the above, except that the supernatants were diluted 1:3 in row A of a 96-well dilution plate, followed by serial 1:3 dilutions in rows B-H and the standard antibody was diluted to a starting concentration of 4 µg/ml in row A followed by successive 1:3 dilutions in rows B-H (Figure 7). In addition, the incubation step with mouse anti-trout (1-14) monoclonal antibody was lengthened to two hours.

FIGURE 5. Schematic diagram of an antibody-capture enzyme-linked immunosorbent assay (ELISA). Antigen molecules coated to wells of a microtiter plate "capture" antibody molecules in the sample being assayed. The excess unbound antibody is removed during washing and the amount of antibody that is bound to the antigen is determined by the addition of a biotinylated secondary antibody. The antibody present is visualized by the colored product formed when strepavidin-conjugated horseradish peroxidase and a substrate solution are added.

Antibody-capture enzyme-linked immunosorbent assay (ELISA).

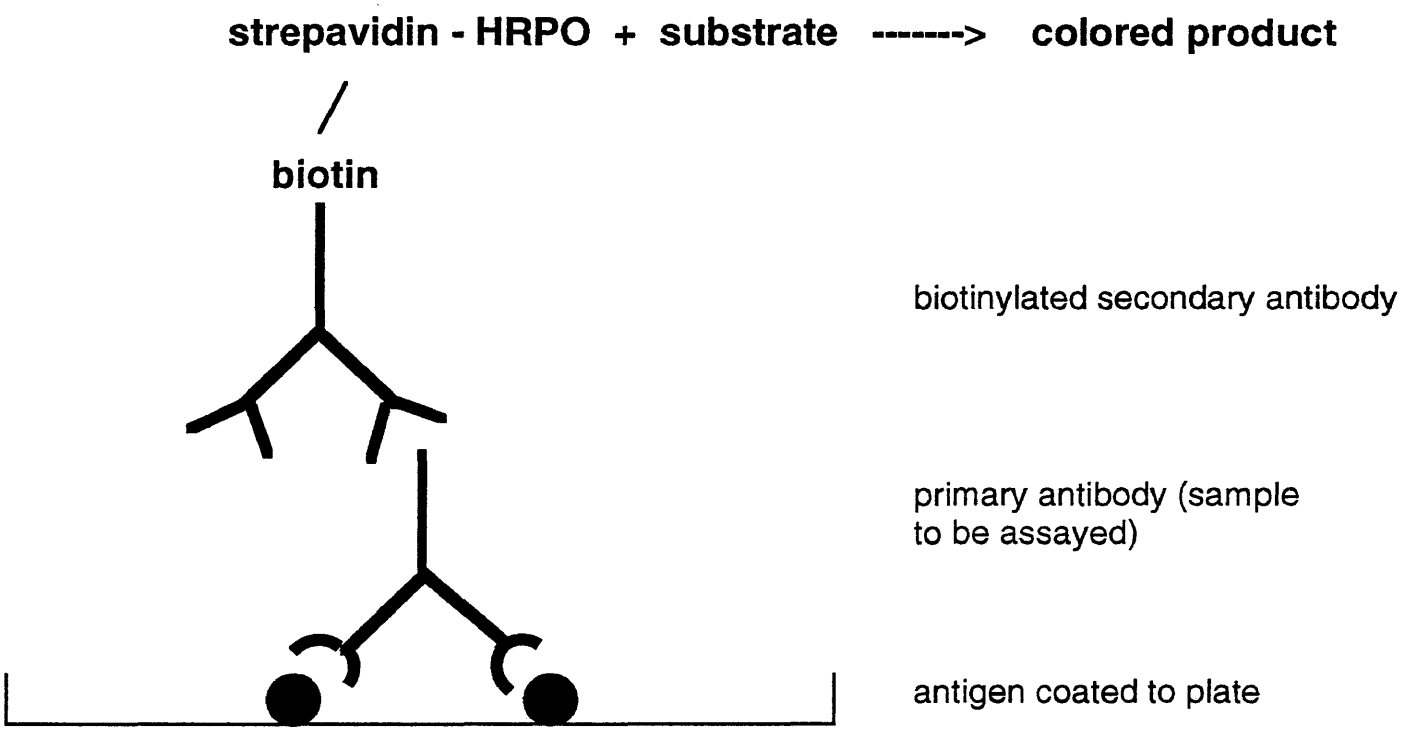


FIGURE 6. Determination of plasma volume containing one unit of antibody activity from plasma titration curves obtained by antibody-capture ELISA. One unit of antibody activity is arbitrarily designated as the amount of antibody required to produce an O.D. equivalent to half of the maximum O.D. attained by the standard antibody titration curve. In this way a standard concentration of antibody (one unit/well) could be used in each affinity ELISA assay.

Determination of volume of plasma containing one unit of antibody activity.

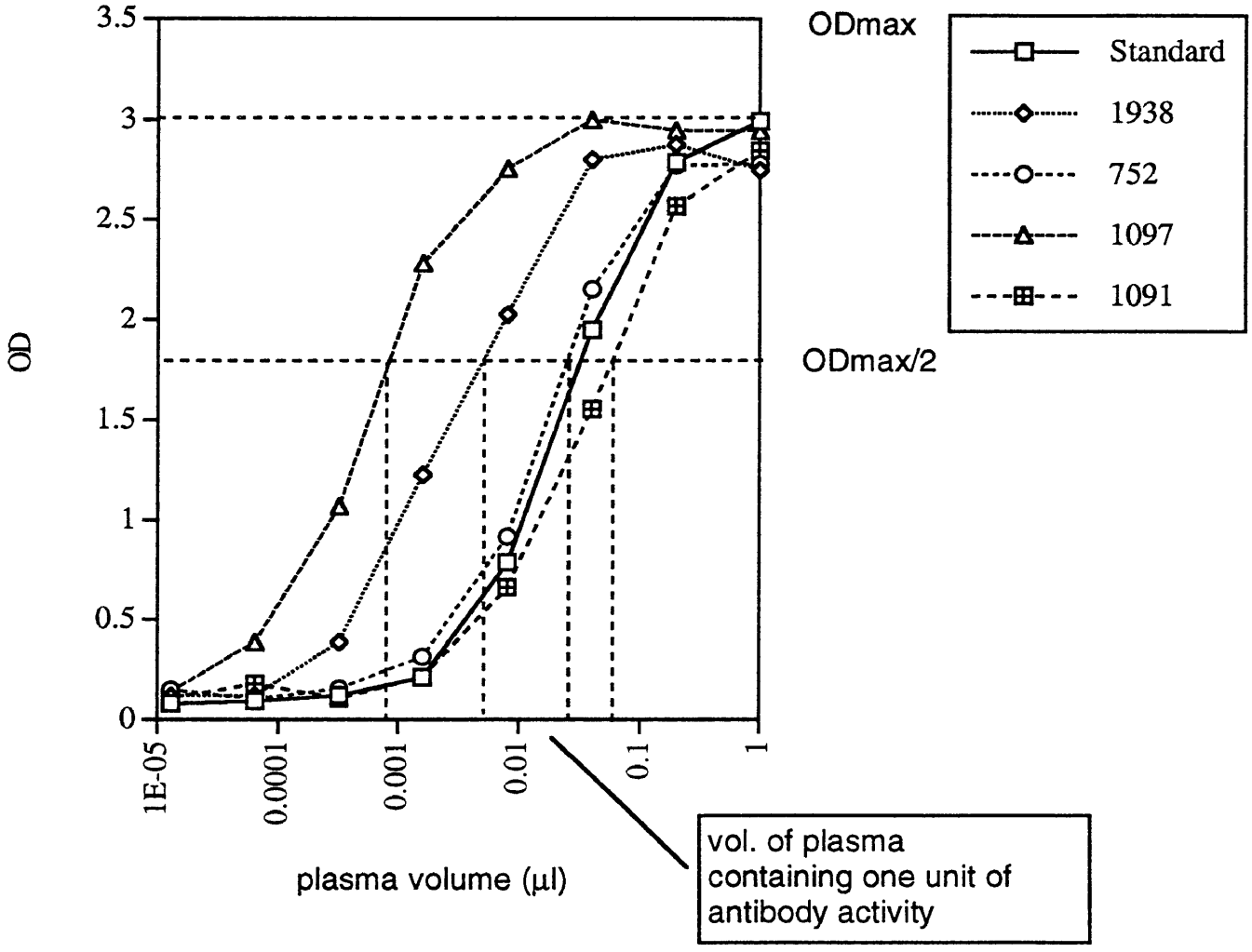
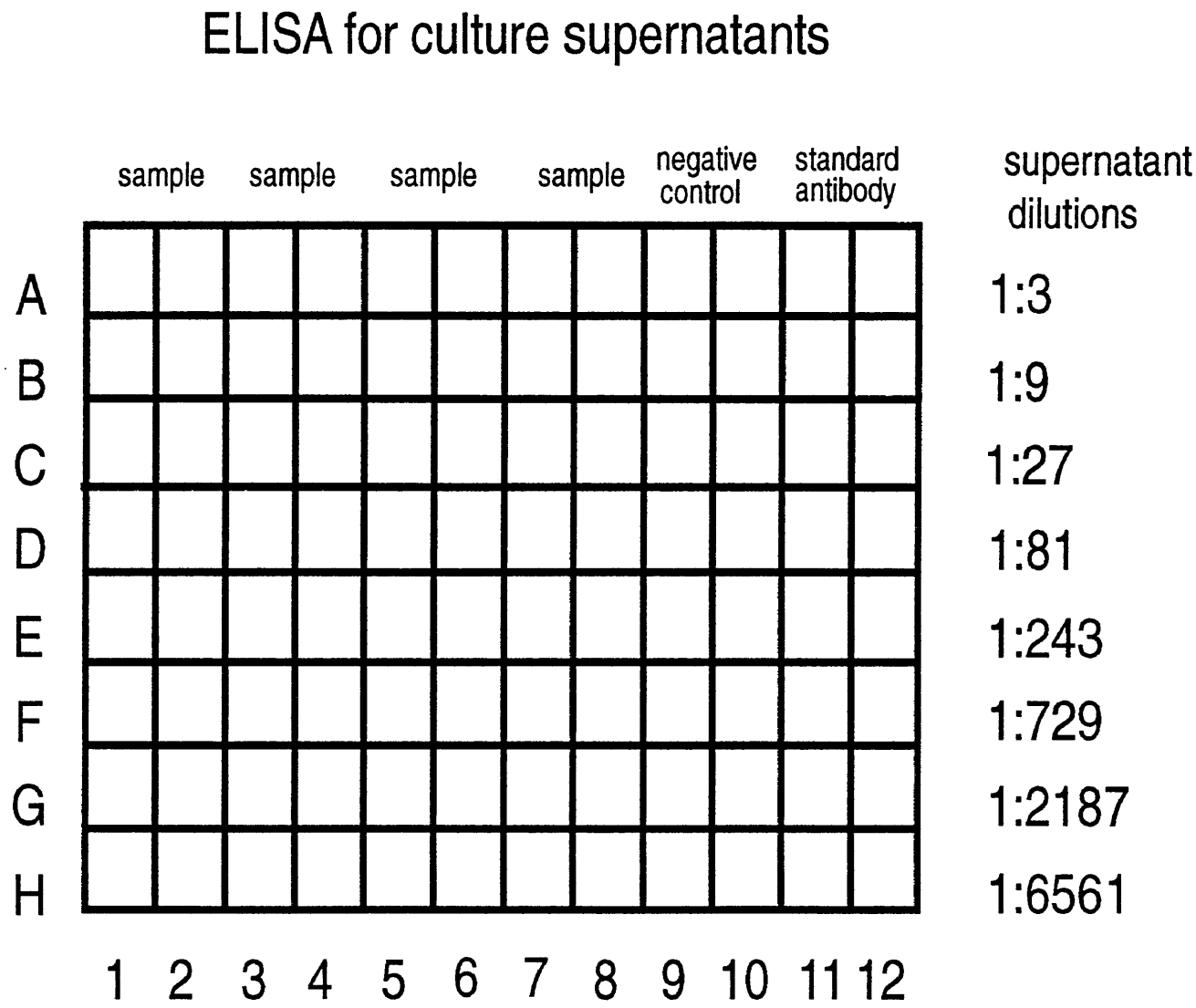


FIGURE 7. ELISA plate design for assaying concentration of antibody in culture supernatants. Antigen is coated onto the wells of a microtiter plate and following blocking of the wells, serial 1:3 dilutions of the supernatant are made and placed in duplicate wells. A standard antibody is initially diluted to a concentration of 4 μg/ml and placed in duplicate wells in row A, followed by serial 1:3 dilutions in rows B-H. Buffer is placed in duplicate columns to serve as a negative control.

ELISA plate design for assaying concentration of antibodies in culture supernatants.

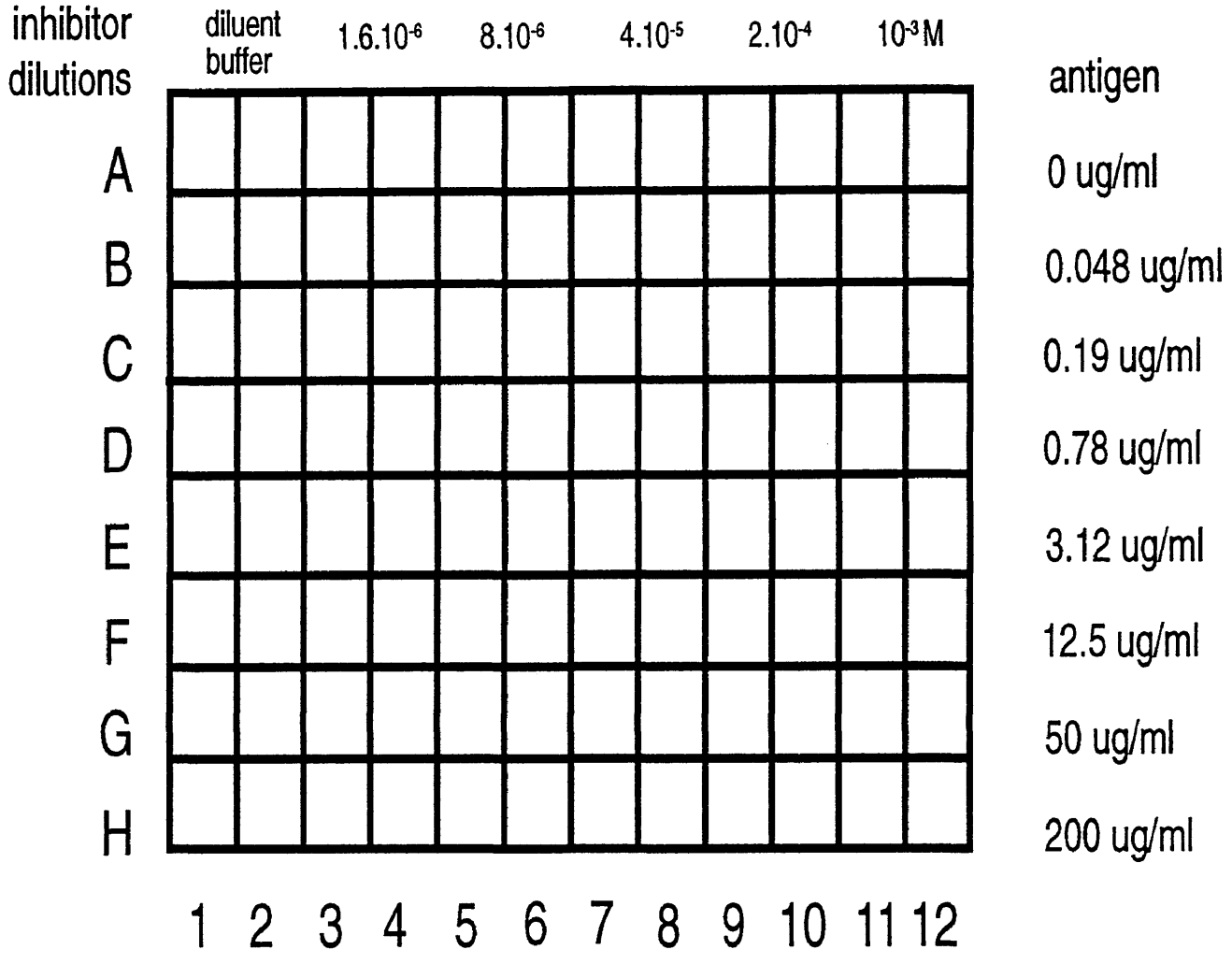


Determination of antibody affinity

The affinity of the antibodies in the plasma obtained at the various time points was determined by a solid-phase inhibition ELISA method (Kaattari and Shapiro, 1994; Nieto et al., 1984; Van Dam et al., 1989). This involves coating row A (Figure 8) of a 96-well microtiter plate with 50 µl/well of a starting concentration of 200 µg/ml of the multivalent antigen TNP-BSA in coating buffer, followed by successive 1:4 dilutions in rows B-H, for 1 hour at room temperature or overnight at 17°C. Row A receives only coating buffer and thus serves as a negative control. Following incubation with antigen the plate was washed three times with TTBS and blocked with 240 µl/well of 1% bovine serum albumin (BSA) in TTBS for one hour at room temperature. The blocking agent was then removed and the plate was washed three times with TTBS. Serial 1:5 dilutions were made of the inhibitor, the monovalent antigen TNP-lysine, and 50 µl/well of each inhibitor dilution was loaded onto duplicate columns of wells, with the first two rows receiving only TTBS, the diluent buffer. The plasma was suitably diluted so as to dispense one unit of antibody (as determined by antigen-capture ELISA) in 50 µl to each well. The plate was then incubated with inhibitor and sample for 90 min at room temperature. After incubation the plate was washed three times with TTBS and 100 µl/well of biotinylated monoclonal mouse anti-trout antibody (1-14) was added and the plate was again incubated for one hour. The plate was washed three times with TTBS and the previous step was repeated with strepavidin-conjugated horseradish peroxidase (SA-HRPO). In the final step the plate was washed five times and incubated with 100 µl/well of substrate solution as described above for 20-30 minutes (Kaattari and Shapiro, 1994). The O.D. values were then determined by means of a *Titertek Multiskan* MCC/340 microplate reader. The antibody mixture was partitioned into seven distinct affinity subpopulations by the different concentrations of coated antigen.

FIGURE 8. Affinity ELISA plate design for determination of affinity distributions of antibodies. An initial concentration of 200 μg/ml of the antigen TNP-BSA is used to coat the wells in row H of a 96-well microtiter plate. This is followed by serial 1:4 dilutions of antigen in rows G up to B. Row A receives no antigen and serves as a negative control. An initial concentration of 10^{-3} M of the inhibitor TNP-lysine is placed in the last two wells of each row, followed by serial 1:5 dilutions in duplicate wells going from right to left of each row. The different antigen concentrations partition antibodies into subpopulations based on their affinity for the antigen and the inhibitor titrations are used to determine the affinity of the antibody subpopulation in each row.

Affinity ELISA plate design for determining antibody affinity distributions.



The affinity of each of these subpopulations was measured using the equation:

$$K = - \log(1/[H]_{50})$$

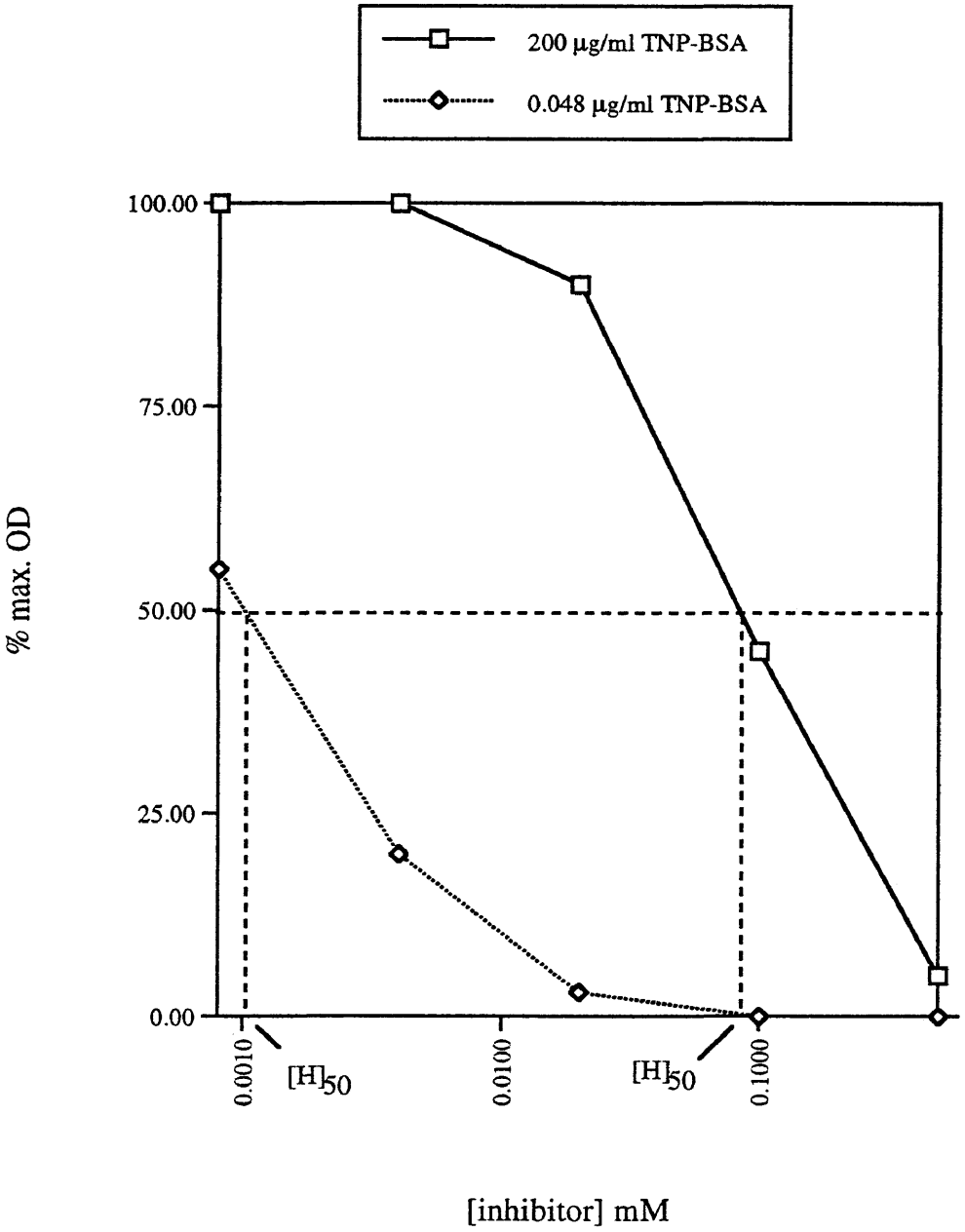
where K is the affinity constant or the measure of the degree of binding between an antibody and its specific antigen and H_{50} is the concentration of hapten (TNP-lysine) required to cause 50% inhibition of the maximum O.D., which is the average O.D. observed in the first two wells of each row of an affinity ELISA plate (Nieto *et al.*, 1984; Kaattari and Shapiro, 1994). Figure 9 is a plot of O.D. versus inhibitor concentration and demonstrates how the affinity K can be determined graphically. A weighted average affinity of all the antibody subpopulations can be calculated from the K values and the proportion of antibody in each subpopulation using the equation:

$$\text{weighted average affinity} = \sum (K \times P)$$

where K is the affinity of a particular subpopulation and P is the fraction of total antibody in that subpopulation (Kaattari and Shapiro, 1994). Alternatively, the affinity and proportion of antibody in each subpopulation as well as the weighted average affinity can be calculated using a macro on *Microsoft Excel* software.

FIGURE 9. Determination of affinity K from a plot of O.D. versus inhibitor concentration. $K = - \log(1/[H]_{50})$ where $[H]_{50}$ is the concentration of inhibitor required to inhibit 50% of the binding of antibody to antigen, i.e. to produce half of the maximum O.D. The affinity K can be determined for each of the seven antibody subpopulations obtained with the affinity ELISA. Only the titer curves for the subpopulations bound by the highest and lowest concentration of antigen are shown for simplicity.

Determination of affinity K from a plot of OD versus concentration of inhibitor.



Statistical tests

In order to determine if the two groups of fish, normal and AFB1-exposed, differed in their antibody titers and average affinities, an unpaired non-parametric Mann-Whitney U test was conducted comparing the two groups at each of the time points. The null hypothesis for the test comparing the titers was that there was no difference between the antibody titers of the two groups at each time point. The null hypothesis for the test comparing the average affinities was that there was no difference between the average affinities of the two groups.

The change in skewness indices of the affinity distributions between week 0 and week 5, week 5 and week 12, week 12 and week 21 and between week 21 and week 25 was tested for significance at the 5% confidence level using a non-parametric paired test, the paired sign test, to compare the skewness index of each fish at one time point with its skewness index at the previous time point. The null hypothesis for each pair of time points is that there is an equal probability that the value would be higher or lower at a particular time point than at the time point preceding it.

RESULTS

In vivo antibody titers and affinity distributions

Two groups of rainbow trout, one group that had been embryologically exposed to aflatoxin B1 (referred to as "AFB1") and was comprised of 12 fish, and 15 non-exposed controls (referred to as "normals"), were immunized with TNP-KLH and boosted at week 21. The plasma antibody titers obtained at weeks 0, 5, 12, 21 and 25 post primary immunization were determined by an antibody-capture ELISA (Table 1). The titers are expressed in units of antibody activity/ml plasma. A solid-phase affinity ELISA (Kaattari and Shapiro, 1994) was used to partition each plasma sample into its component antibody subpopulations, each with a distinct affinity. The weighted average affinities for the two groups of fish at weeks 0, 5, 12, 21 and 25 post primary immunization are shown in Table 2. In order to determine if there was a difference in the quantifiable immune response between the two groups of fish with respect to the antibody titers and affinities, unpaired non-parametric Mann-Whitney U tests were performed to compare the antibody titers and average affinities of the two groups using *Abacus Statview 4.5* software. Due to the large variability of the titers between individuals in the same group and the presence of outliers, the non-parametric Mann-Whitney U test was used instead of an unpaired t-test. The titers of all the normal fish were compared against the titers of all the AFB1 fish at each of the five time points, with the null hypothesis at each time point stating that there was no difference between the two groups. The results of the five Mann-Whitney U tests are summarized in Table 3. The p values obtained were 0.4068 at week 0, 0.5458 at week 5, 0.6310 at week 12, 0.6547 at week 21 and 0.8815 at week 25. Thus at each time point the p value was large and the null hypothesis could not be rejected, indicating that there was essentially no difference between the two groups. The mean antibody titers for each group at each time point were also plotted on a graph of mean titer versus time (Figure 10). At

each time point the error bars for the normal and AFB_1 mean titers overlapped, providing further evidence for the lack of difference between the groups. However, due to the presence of an outlier at week 12, the standard error for the mean titer at week 12 was very large, possibly giving an inaccurate result. The mean titers were thus plotted against time with the exclusion of this outlier (Figure 11). The error bars at each time point overlapped and the curves for the two groups were in fact more similar. An unpaired t-test was conducted to compare the antibody titers at week 12 of the normal group with those of the AFB_1 group with the exclusion of the outlier. The p value was 0.319 and once again the null hypothesis could not be rejected, indicating no significant difference between the groups even with exclusion of the outlier.

The average affinities of the two groups were likewise compared by a Mann-Whitney U test at each time point, with the null hypothesis stating that there was no difference between the average affinities of the two groups. The results of the five Mann-Whitney U tests are summarized in Table 4. The p values obtained were 0.9394 at week 0, 0.0829 at week 5, 0.7150 at week 12, 0.8815 at week 21 and 0.3173 at week 25. Once again the p values were > 0.05 and the null hypothesis could not be rejected, indicating that at each point in time the average affinities of the two groups were not significantly different. The mean average affinities for each group were plotted for each time point (Figure 12) and the error bars of the normal and AFB_1 mean average affinities overlapped at all the time points except for week 5. Based on results of the above statistical tests, it was inferred that the two groups were not significantly different in their quantifiable antibody response and could be pooled to increase the accuracy of the experiments. This was desirable since mortalities occurred during the course of the study, decreasing the number of subjects at the later time points. The fish were thus considered as one group in the following analyses. The mortalities occurred equivalently in both treatments, indicating that they were independent of treatment. If any specifc cause was responsible, it affected both groups equally. The mean titers for the pooled group of fish were plotted against

TABLE 1. Plasma antibody titers. Fifteen normal and twelve AFB_1-exposed fish were given a primary immunization of 800 μg/kg of TNP-KLH in Freunds Complete Adjuvant and a secondary immunization of 400 μg/kg of TNP-KLH in Freunds Incomplete Adjuvant at week 21. Blood samples were taken at weeks 0, 5, 12, 21 and 25 post primary immunization. The antibody titers of the plasma were determined by an antibody-capture ELISA in which the standard antibody (0.310 mg/ml protein) was assigned a titer of 1000 units.

Plasma antibody titers.

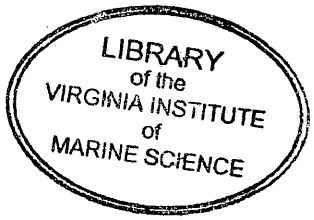
Treatment	Time (weeks)	0	5	12	21	25
Normals	749	170.1	1031.8	593.7	517.5	389.1
	738	135.1	229.1	3462.3	3695.9	2863.3
	734	2.5	139.0	7771.5	5274.8	6864.1
	737	2.3	5.1	35837.7	9708.2	2177.9
	764	129.8	195.1	175.9	373.7	188.9
	735	229.7	270.0	2792.3	-	-
	751	23.0	244.7	-	-	-
	652	33.7	94.9	-	-	-
	744	63.3	546.4	-	-	-
	743	175.9	784.0	-	-	-
	736	2.2	8.0	-	-	-
	677	11.7	-	-	-	-
	745	10.6	44.4	-	-	-
	742	464.0	-	-	-	-
	741	27.1	-	-	-	-
AFB_1 -	752	18.0	33.7	1255.8	2827.9	4365.3
exposed	758	109.2	185.8	1832.0	686.5	893.2
	756	15.4	604.8	3083.4	1479.3	996.5
	767	153.5	781.2	2578.8	-	-
	760	81.8	290.9	5076.7	-	-
	759	12.1	311.0	1442.9	-	-
	757	11.0	61.7	-	-	-
	770	103.0	6443.3	-	-	-
	768	30.4	44.4	-	-	-
	755	11.0	-	-	-	-
	763	16.3	-	-	-	-

TABLE 2. Plasma antibody weighted average affinities. The weighted average affinities were determined for plasma samples at weeks 0, 5, 12, 21 and 25 using the equation: weighted average affinity = $\sum$ (K x P) , where K is the affinity of a particular subpopulation and P is the fraction of the total antibody in that subpopulation.

Plasma weighted average affinities.

Treatment	Time (weeks)	0	5	12	21	25
Normals	749	4.44	4.45	4.85	5.00	5.24
	738	4.33	4.37	4.82	5.09	4.70
	734	4.67	4.77	5.30	5.00	4.68
	737	4.27	4.72	*	5.25	5.25
	764	4.44	4.43	4.56	4.48	4.48
	735	4.70	4.55	5.20	-	-
	751	4.45	4.29	-	-	-
	652	4.99	4.39	-	-	-
	743	4.53	4.61	-	-	-
	736	4.87	4.01	-	-	-
	745	4.64	4.42	-	-	-
AFB_1 -	752	4.89	4.58	5.14	4.97	5.44
exposed	756	4.45	5.00	4.91	5.01	5.40
	758	4.35	4.28	4.83	5.02	4.85
	767	4.41	4.77	5.06	-	-
	760	4.56	4.69	4.95	-	-
	759	4.50	4.39	5.14	-	-
	770	4.68	5.11	-	-	-
	768	4.39	4.79	-	-	-
	755	5.13	-	-	-	-

TABLE 3. Comparison of plasma antibody titers of normal and AFB1-exposed fish at each time point using an unpaired non-parametric Mann-Whitney U test. At each time point, the null hypothesis states that there is no difference in antibody titer between the two groups. The p values at each time point are high (>>0.05), indicating that there is no difference between the groups at the 5% confidence level.



Comparison of antibody titers of normal and AFB_1 fish by Mann-Whitney U tests

Time Point	Description	No. fish	Sum Ranks	Mean Rank	Mean Titer	Std. Error	p value
week 0	Normal	12	151.000	12.583	98.7	32.6	0.4068
	AFB1	15	227.000	15.133	46.9	14.7	
week 5	Normal	9	107.500	11.944	299.4	93.6	0.5458
	AFB1	12	123.500	10.292	973.0	689.1	
week 12	Normal	6	36.000	6.000	8438.9	5590.5	0.6310
	AFB1	6	42.000	7.000	2544.9	579.8	
week 21	Normal	3	12.000	4.000	3914.0	1725.4	0.6547
	AFB1	5	24.000	4.800	1664.6	625.1	
week 25	Normal	3	14.000	4.667	2496.7	1205.8	0.8815
	AFB1	5	22.000	4.400	2085.0	1140.5	

FIGURE 10. Plot of mean titers for normal and AFB_1-exposed fish over time. At each time point the error bars of the mean titers overlap. Vertical bars represent mean ± standard error.

Comparison of mean titers for normal and AFB_1 fish.

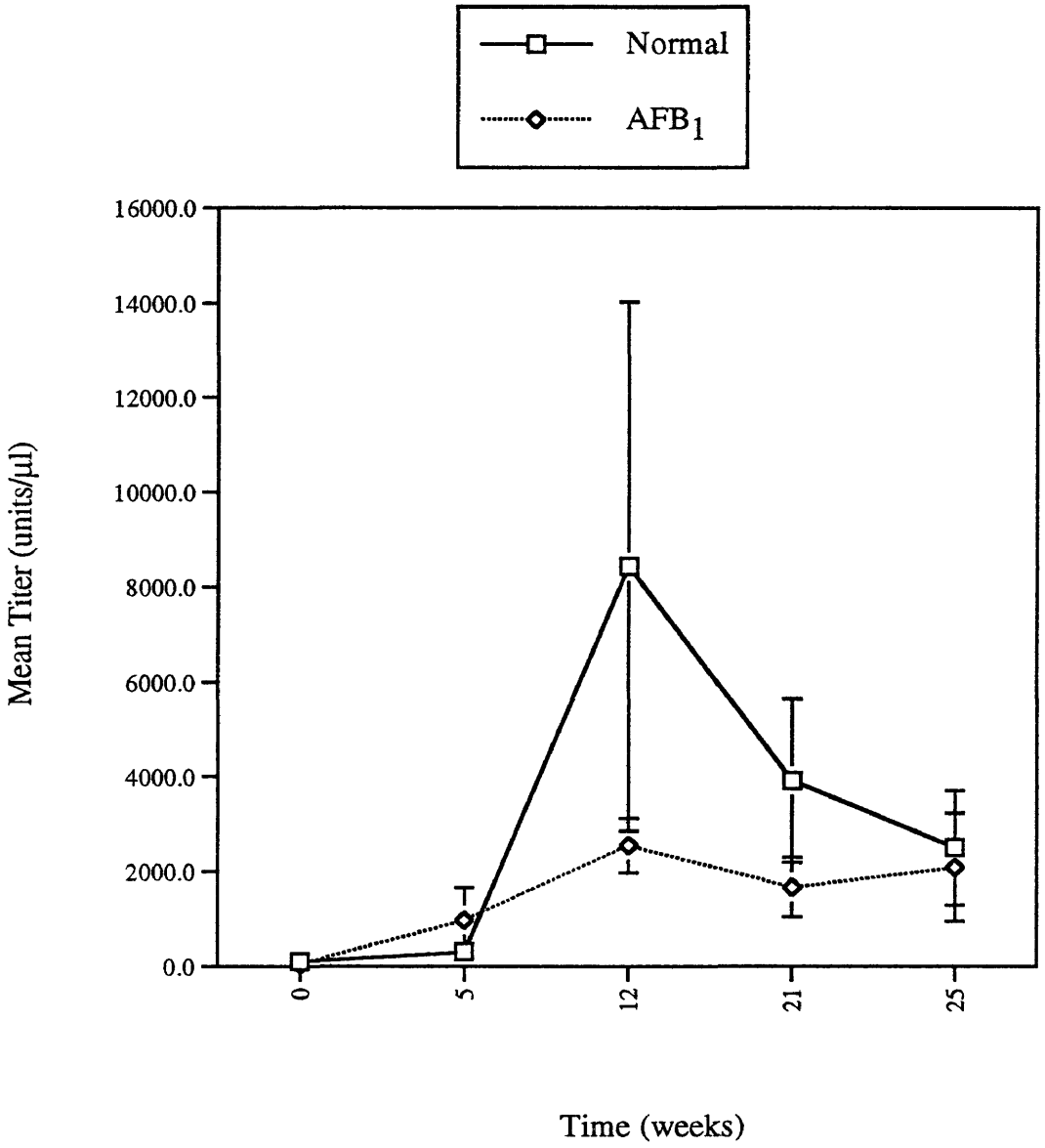


FIGURE 11. Plot of mean titers for normal and AFB_1-exposed fish over time with the exclusion of an outlier (#737) at week 12. At each time point the error bars of the mean titers overlap. Vertical bars represent mean ± standard error.

Comparison of mean titers of normal and AFB_1 fish excluding an outlier at week 12.

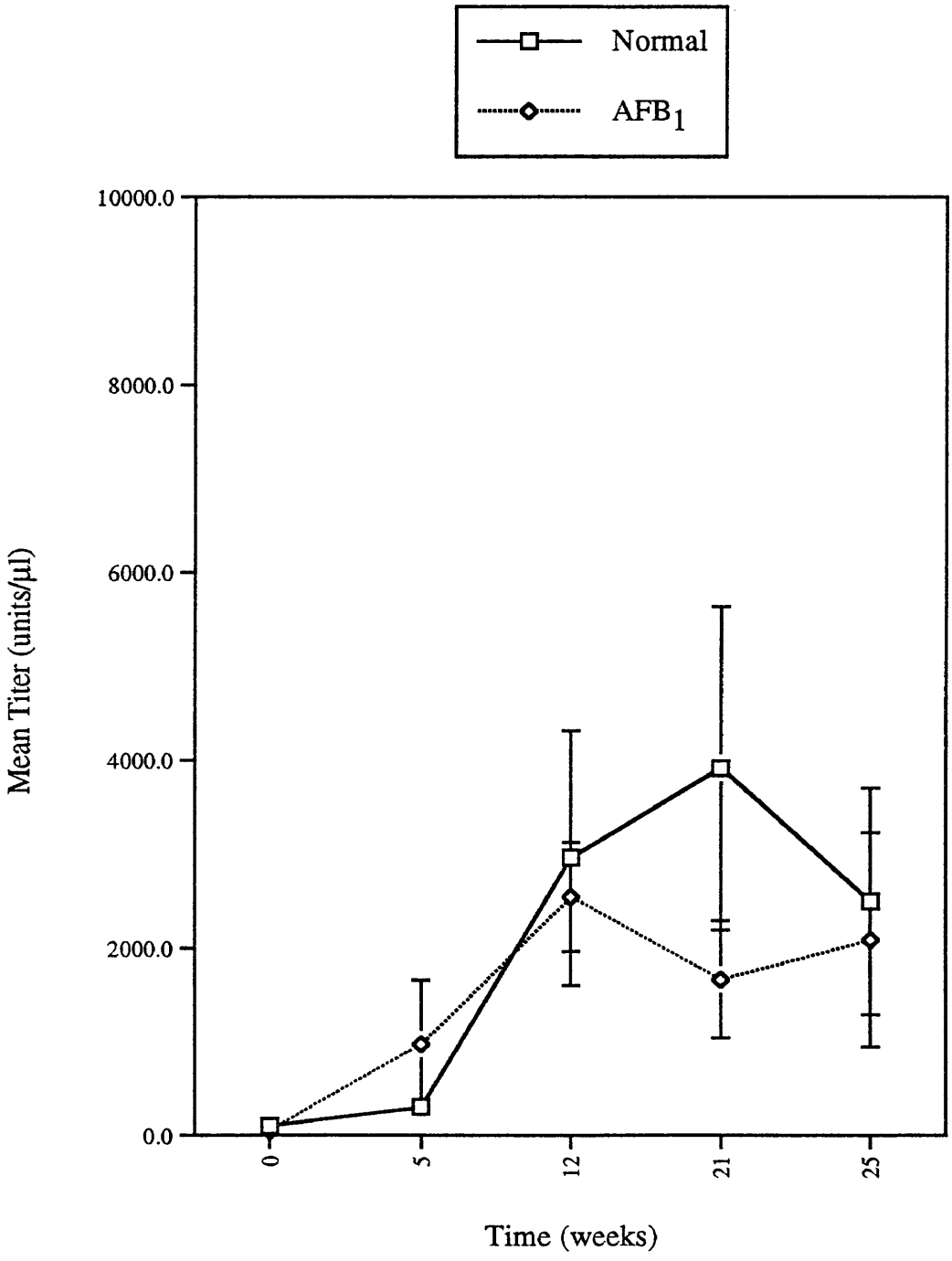


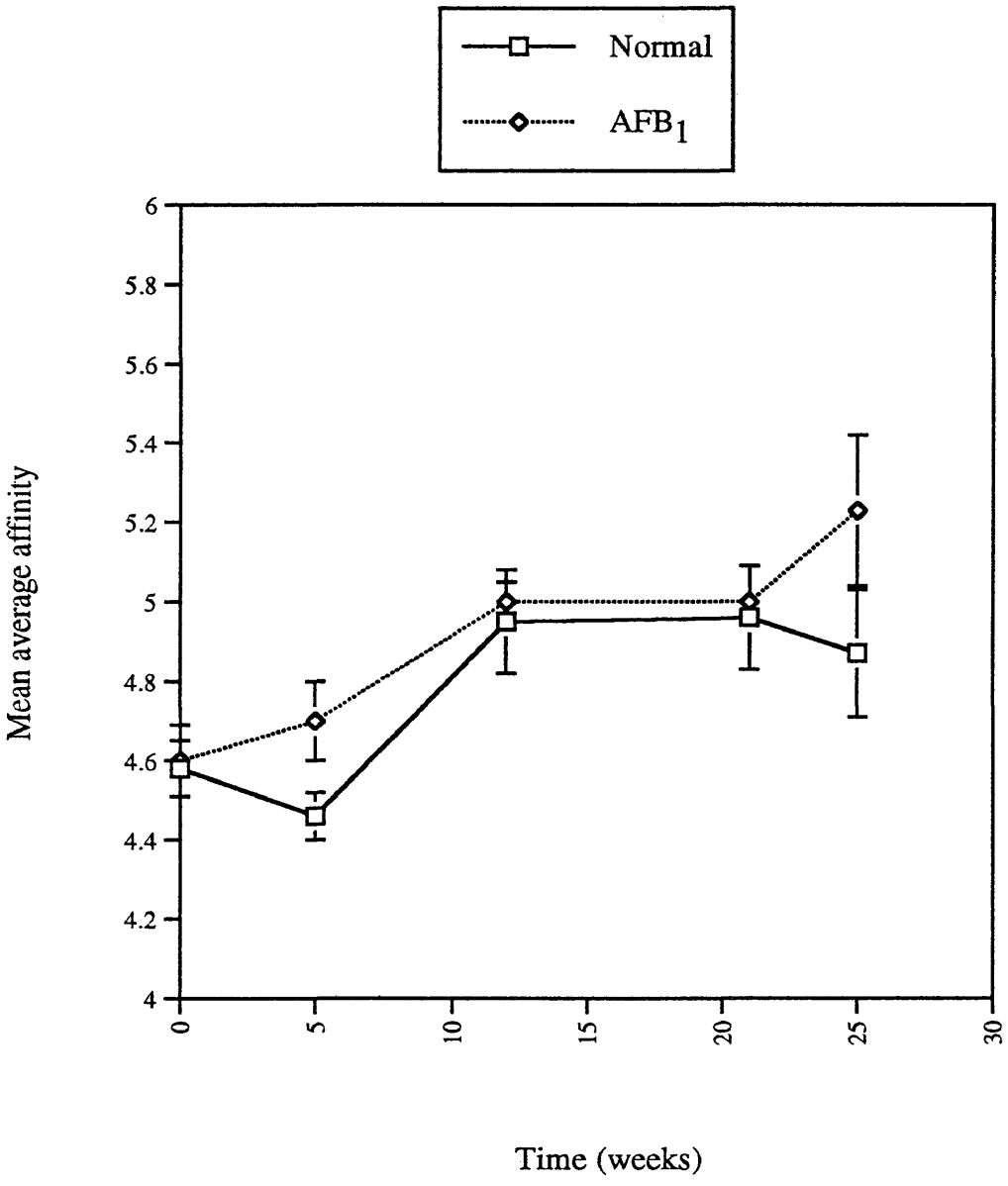
TABLE 4. Comparison of weighted average affinities of normal and AFB_1-exposed fish at each time point using an upaired non-parametric Mann-Whitney U test. At each time point, the null hypothesis states that there is no difference in weighted average affinity between the two groups. The p values at each time point are >0.05, indicating that there is no difference between the groups at the 5% confidence level.

Comparison of average affinities of normal and AFB_1 fish using Mann-Whitney U tests.

Time Point	Description	No. fish	Sum Ranks	Mean Rank	Mean K	Std. Error	p value
week 0	Normal	9	95.500	10.611	4.58	0.07	0.9394
	AFB1	11	114.500	10.409	4.60	0.09	
week 5	Normal	8	101.000	12.625	4.46	0.06	0.0829
	AFB1	11	89.000	8.091	4.70	0.10	
week 12	Normal	6	38.000	6.333	4.95	0.13	0.7150
	AFB1	5	28.000	5.600	5.00	0.05	
week 21	Normal	3	13.000	4.333	4.96	0.13	0.8815
	AFB1	5	23.000	4.600	5.00	0.02	
week 25	Normal	1	2.000	2.000	4.87	0.16	0.3173
	AFB1	1	1.000	1.000	5.23	0.19	

FIGURE 12. Plot of mean weighted average affinities for normal and AFB_1-exposed fish over time. At each time point the error bars of the mean weighted average affinities overlap. Vertical bars represent mean ± standard error.

Comparison of mean average affinities of normal and AFB_1 fish.



time (Figure 13). The graph shows an increase in mean titer from week 0 to week 21 and then a drop from week 21 to week 25. The mean average affinities for the pooled group of fish were also plotted against time (Figure 14). The mean average affinities decreased slightly from week 0 to week 5, then increased dramatically from week 5 to week 12. After week 12 there was little change in mean average affinity.

The antibody titers were also monitored over time for the individual fish. Considering the data in Table 1, a significant antibody response was induced. The antibody titers for all individuals increased from week 0 to week 5 post primary immunization. Eight individuals were monitored through week 25 and an additional four through week 12. Out of the eight fish monitored through week 25, one individual (#764) did not appear to establish an immune response to the antigen, as evidenced by the that fact that its antibody titer remained relatively constant throughout. In five out of the other seven individuals, antibody titer increased from week 0 to week 12 and then dropped from week 12 to week 21. For one of the remaining individuals (#738), the antibody titer continued to increase slightly from week 12 to week 21. The titer in the other fish (#749) increased from week 0 to week 5 and then decreased by week 12. In five out of eight individuals, the antibody titer decreased slightly from week 21 to week 25 following secondary immunization. In the other three individuals the antibody titer increased slightly post secondary immunization. In the four individuals that were followed from week 0 through week 12, the antibody titers increased steadily following primary immunization. Thus, in the majority of the subjects the antibody titer peaked at week 12 and declined by week 21. After secondary immunization (week 25), the titers in most of the fish were lower, but only slightly so, than at the end of the primary response (week 21).

A solid-phase affinity ELISA (Kaattari and Shapiro, 1995) was used to partition each plasma sample into its component antibody subpopulations, each with a distinct affinity. The affinity distribution for each plasma sample was plotted showing the relative percentage of antibody in each antibody subpopulation. The affinity distributions of

FIGURE 13. Plot of mean titers against time for the pooled group of fish. Vertical bars represent mean ± standard error.

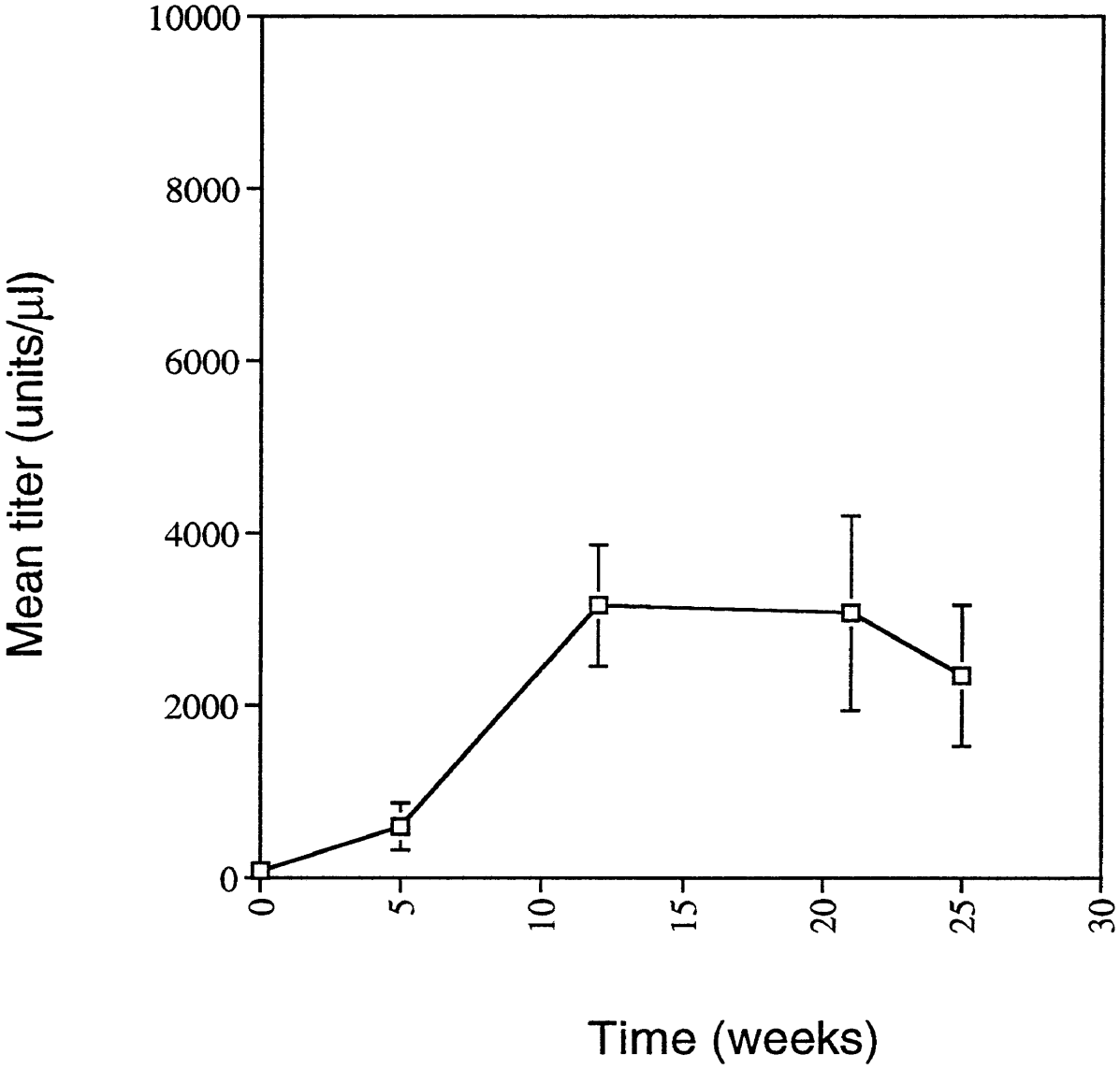
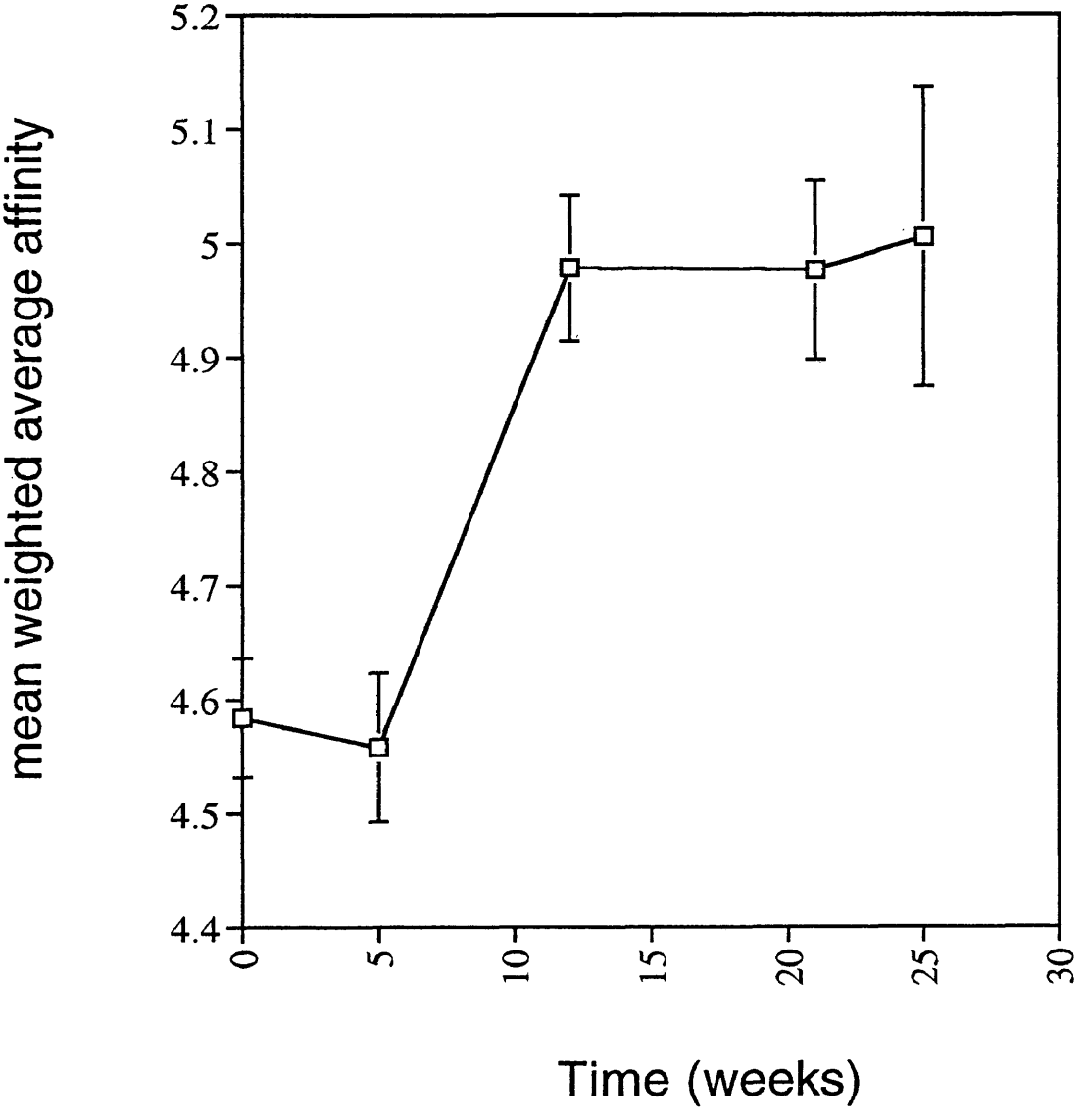

10000
8000
6000
4000
2000
0
Mean titer (units/µl)
0
5
10
15
20
25
30
Time (weeks)

FIGURE 14. Plot of mean weighted average affinities against time for the pooled group of fish. Vertical bars represent mean ± standard error.

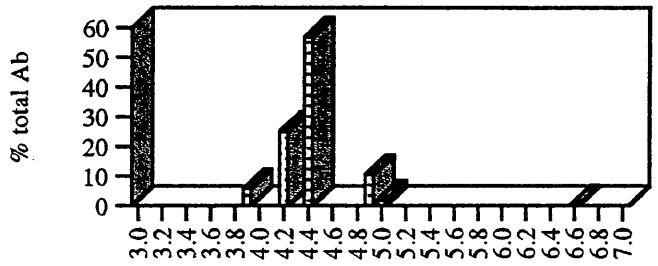

mean weighted average affinity
5.2
5.1
5
4.9
4.8
4.7
4.6
4.5
4.4
0
5
10
15
20
25
30
Time (weeks)

antibodies in plasma obtained at weeks 0, 5, 12, 21 and 25 were compared separately for each individual. Figures 15-22 show the change in the affinity distribution profiles over time for eight individuals. From week 0 to week 21, there is a shift in the affinity distribution from one that has a predominance of low affinity antibodies (week 0 and week 5) to one that is becoming progressively more predominant in high affinity antibodies (week 12, week 21 and week 25). The weighted average affinity for each plasma sample was determined (see Materials and Methods) and the data show that there was an increase in average affinity of the antibodies with time from week 0 to week 21, with a maximum 10-fold increase.

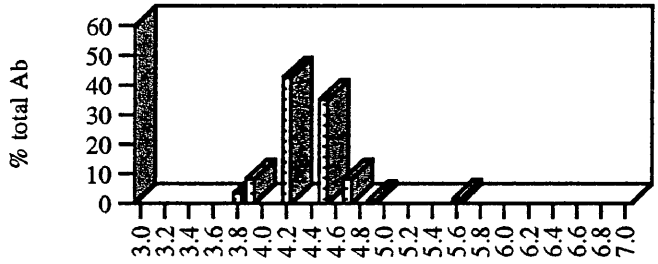
The skewness index of the affinity distribution at each time point was also examined. The skewness index describes the extent of symmetry or asymmetry of the distribution as well as the direction of asymmetry. A perfectly symmetrical distribution possesses a skewness index of zero. The skewness indices of the antibody distributions were calculated for seven individuals from week 0 through week 25 and for four individuals, for which values were only available from week 0 through week 12 (Table 5). The skewness indices were plotted against time for these individuals (Figure 23). The fact that ten out of eleven individuals demonstrated progressively lower (more negative) values at each subsequent time point indicate that there was a considerable trend or bias towards a more negative skewness with time. The skewness indices for the eleventh individual (#764) remained relatively constant and positive throughout. To determine if this trend toward negative skewness was statistically significant, a paired non-parametric test, the paired sign test, was employed. Each paired sign test compared the skewness indices of a group of fish at a particular time point with the skewness indices of a group of fish at the previous time point, considering the skewness indices of each fish at the two different time points in pairs. In other words, a paired sign test was used to compare the skewness indices of the fish at week 0 with the skewness indices at week 5, another test compares the data at week 5 with that at week 12, and so on. The null hypotheses were that the

FIGURE 15. Affinity distributions of plasma antibodies at weeks 0, 5, 12, 21 and 25 for fish #749. Antibodies are partitioned into affinity subpopulations and the percentage of total antibody in each subpopulation is determined. There is a shift from a predominance of low affinity antibodies early in the immune response to a predominance of higher affinity antibodies as time progresses.

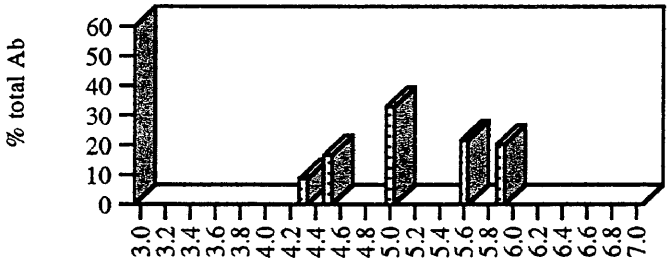
Affinity distributions over time for fish #749. The abscissa is the affinity K and the ordinate is % total antibody.



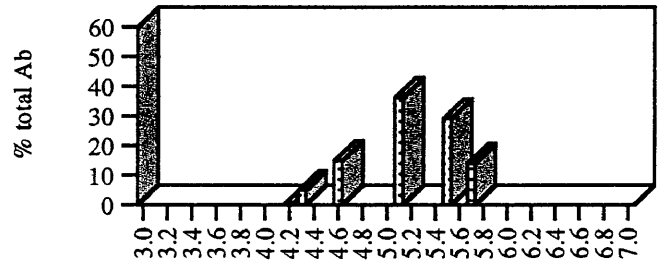
week 0



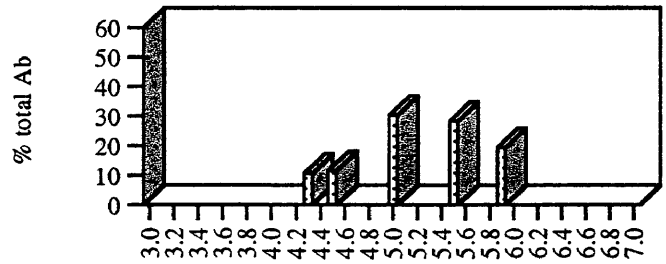
week 5



week 12



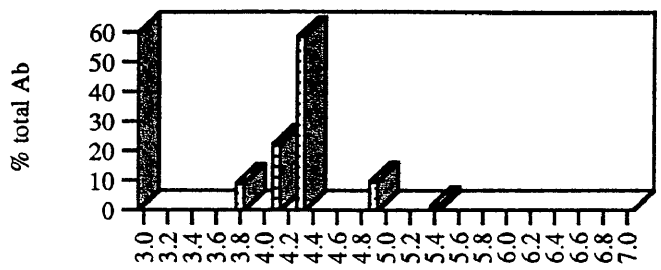
week 21



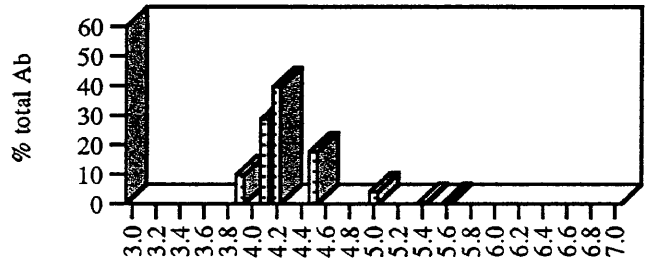
week 25

FIGURE 16. Affinity distributions of plasma antibodies at weeks 0, 5, 12, 21 and 25 for fish #758.

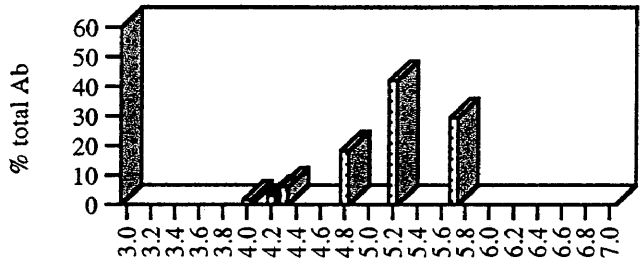
Affinity distributions over time for fish #758. The abscissa is the affinity K and the ordinate is % total antibody.



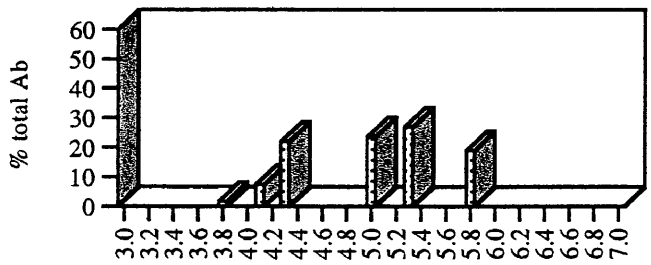
week 0



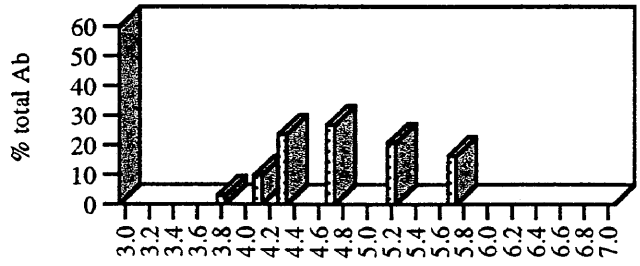
week 5



week 12



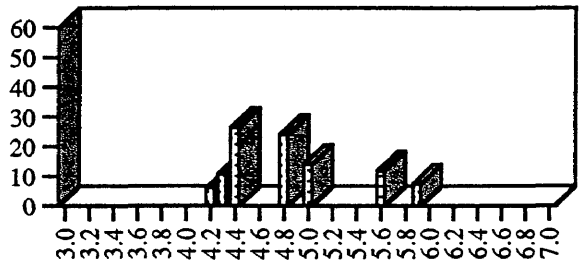
week 21



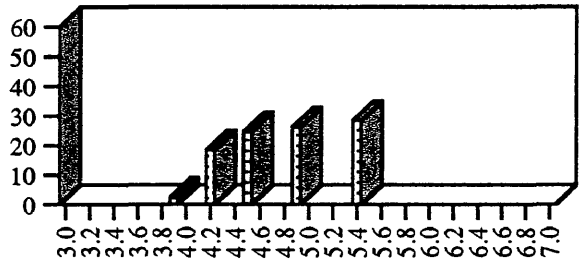
week 25

FIGURE 17. Affinity distributions of plasma antibodies at weeks 0, 5, 12, 21 and 25 for fish #756.

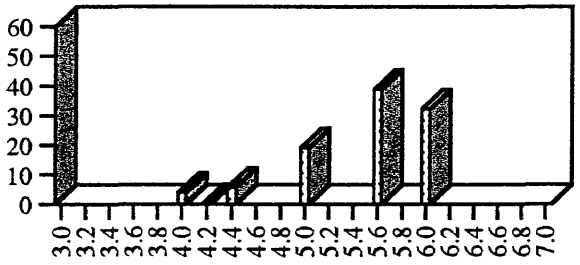
Affinity distributions over time for fish #756. The abscissa is the affinity K and the ordinate is % total antibody.



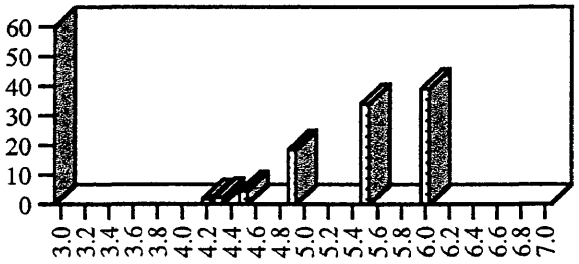
week 0



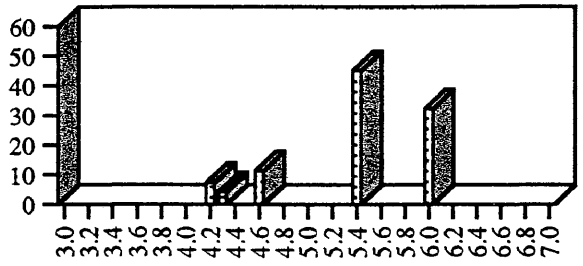
week 5



week 12



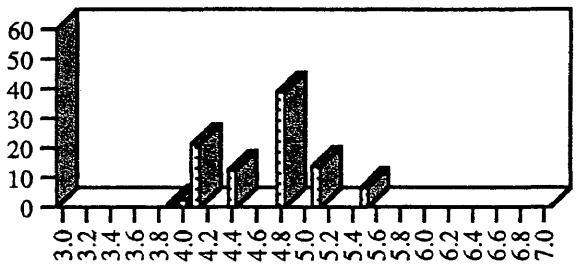
week 21



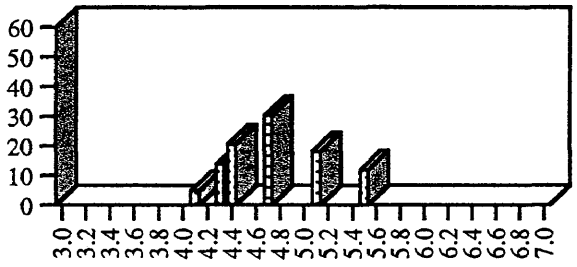
week 25

FIGURE 18. Affinity distributions of plasma antibodies at weeks 0, 5, 12, 21 and 25 for fish #734.

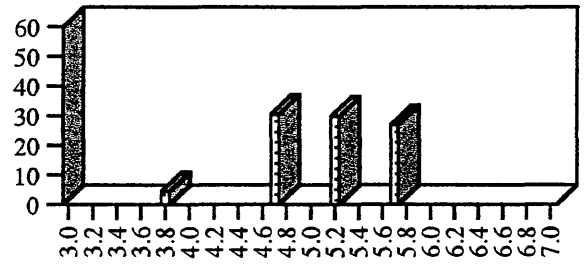
Affinity distributions over time for fish #734. The abscissa is the affinity K and the ordinate is % total antibody.



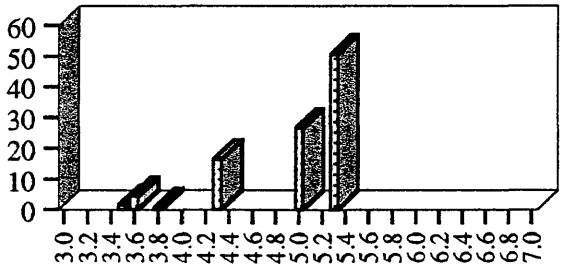
week 0



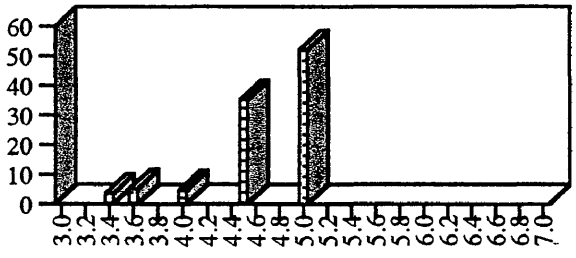
week 5



week 12



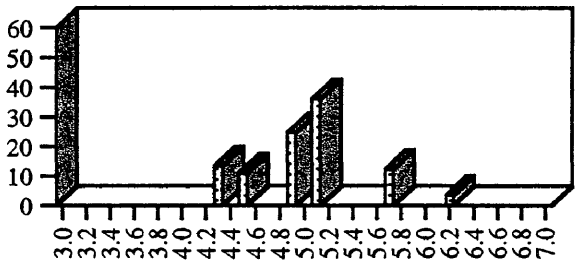
week 21



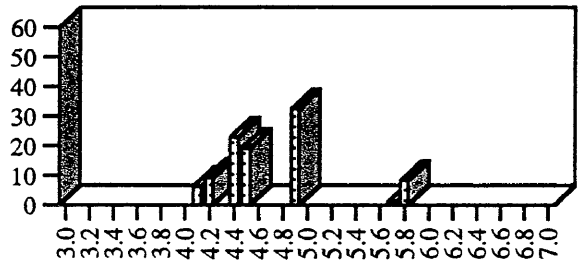
week 25

FIGURE 19. Affinity distributions of plasma antibodies at weeks 0, 5, 12, 21 and 25 for fish #752.

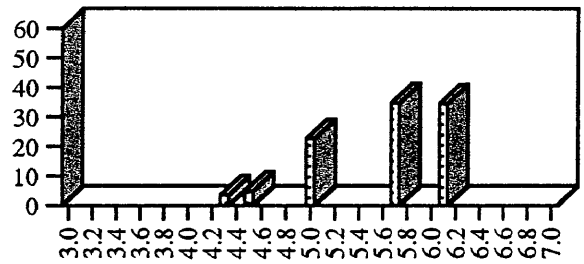
Affinity distributions over time for fish #752. The abscissa is the affinity K and the ordinate is % total antibody.



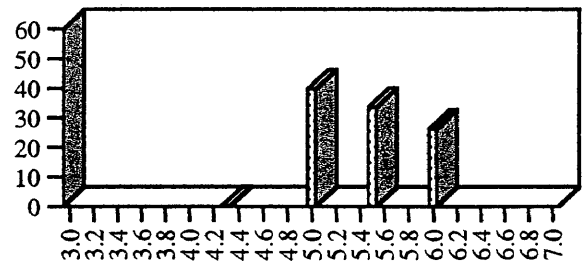
week 0



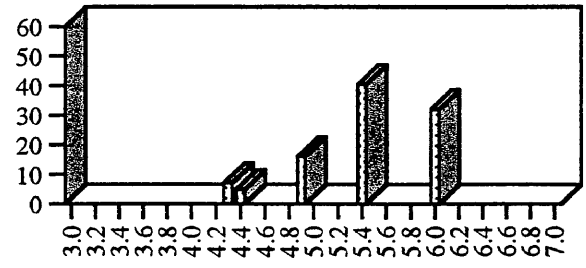
week 5



week 12



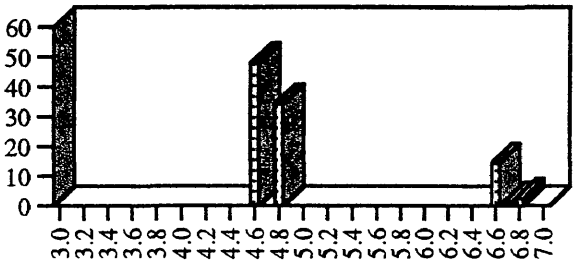
week 21



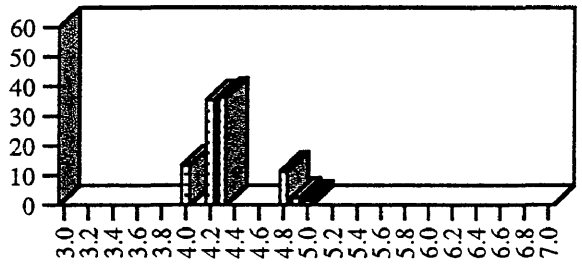
week 25

FIGURE 20. Affinity distributions of plasma antibodies at weeks 0, 5, 12, 21 and 25 for fish #738.

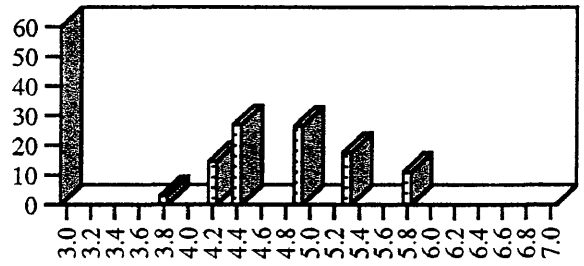
Affinity distributions over time for fish #738. The abscissa is the affinity K and the ordinate is % total antibody.



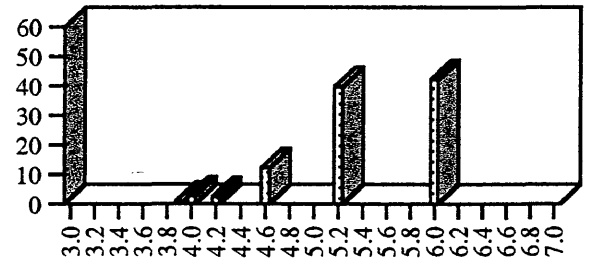
week 0



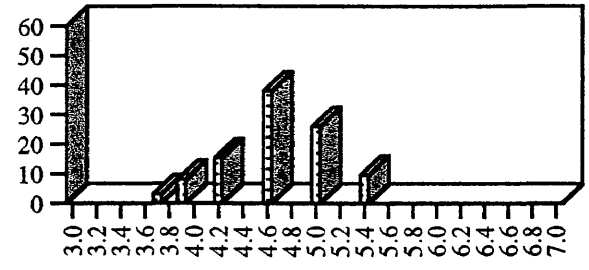
week 5



week12



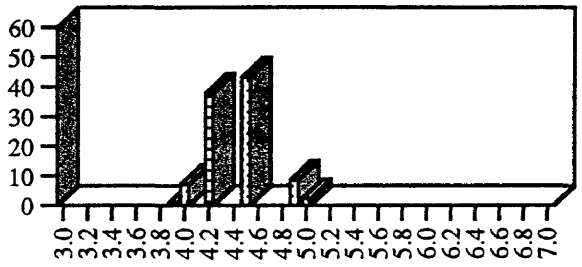
week 21



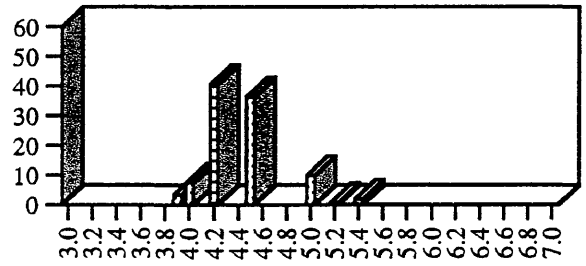
week 25

FIGURE 21. Affinity distributions of plasma antibodies at weeks 0, 5, 12, 21 and 25 for fish #764.

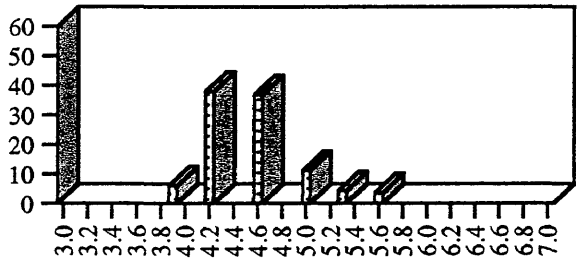
Affinity distributions over time for fish #764. The abscissa is the affinity K and the ordinate is % total antibody.



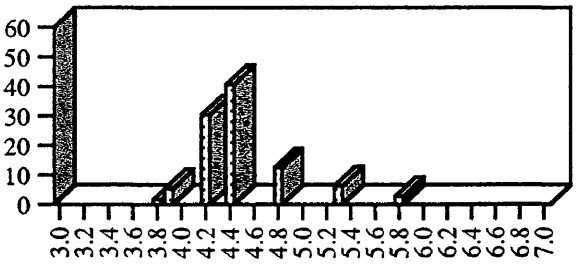
week 0



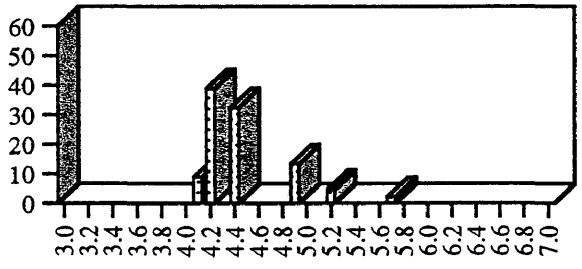
week 5



week 12



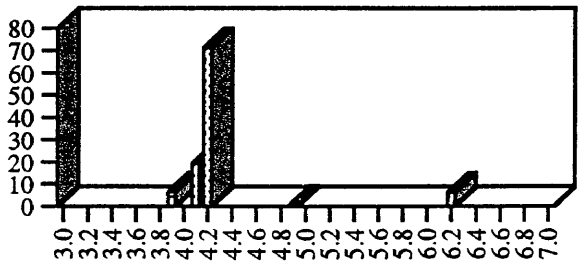
week 21



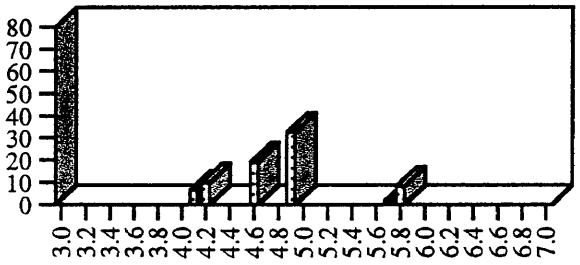
week 25

FIGURE 22. Affinity distributions of plasma antibodies at weeks 0, 5, 12, 21 and 25 for fish #737.

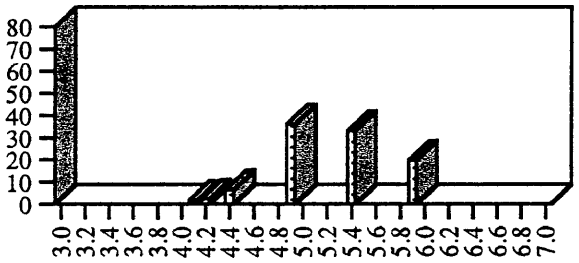
Affinity distributions over time for fish #737. The abscissais the affinity K and the ordinate is % total antibody.



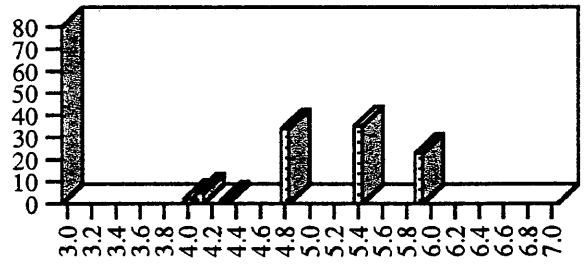
week 0



week 5



week 21*



week 25

*affinity data for week 12 plasma was not obtained due to insufficient antibody

TABLE 5. Skewness indices of plasma affinity distributions. The skewness of each affinity distribution was determined by the equation:

$$\text{skewness} = \frac{\sum (x - xavg)^3/n}{(\sqrt{\sum (x - xavg)^2/n})^3}$$

A positive skewness indicates a predominance of low affinity antibodies and a negative skewness indicates a predominance of high affinity antibodies.

Skewness indices of plasma affinity distributions.

Treatment	Time (weeks) Fish #	0	5	12	21	25
Normals	749	1.24	1.53	-1.19	-0.865	-0.816
	738	1.33	0.700	-1.17	-1.16	-0.783
	734	-0.230	-0.698	-1.12	-1.19	-1.20
	764	0.650	1.01	0.575	1.20	1.42
	735	1.52	0.655	-1.14	-	-
AFB_1 -	756	0.764	-1.01	-1.21	-1.15	-1.09
exposed	752	0.506	1.15	-1.17	-1.17	-1.11
	758	1.80	1.52	-0.824	-1.09	-0.582
	767	1.565	0.661	-0.738	-	-
	760	1.26	0.188	-1.23	-	-
	759	1.61	-0.275	-1.23	-	-

FIGURE 23. Change in skewness index of affinity distributions over time. In ten out of eleven individuals examined, there was a sharp decrease in skewness (i.e. skewness became more negative) from week 5 to week 12. The skewness did not appear to change as drastically before week 5 and after week 12. The skewness for one individual did not change over time but remained positive throughout.

Change in skewness of affinity distributions over time

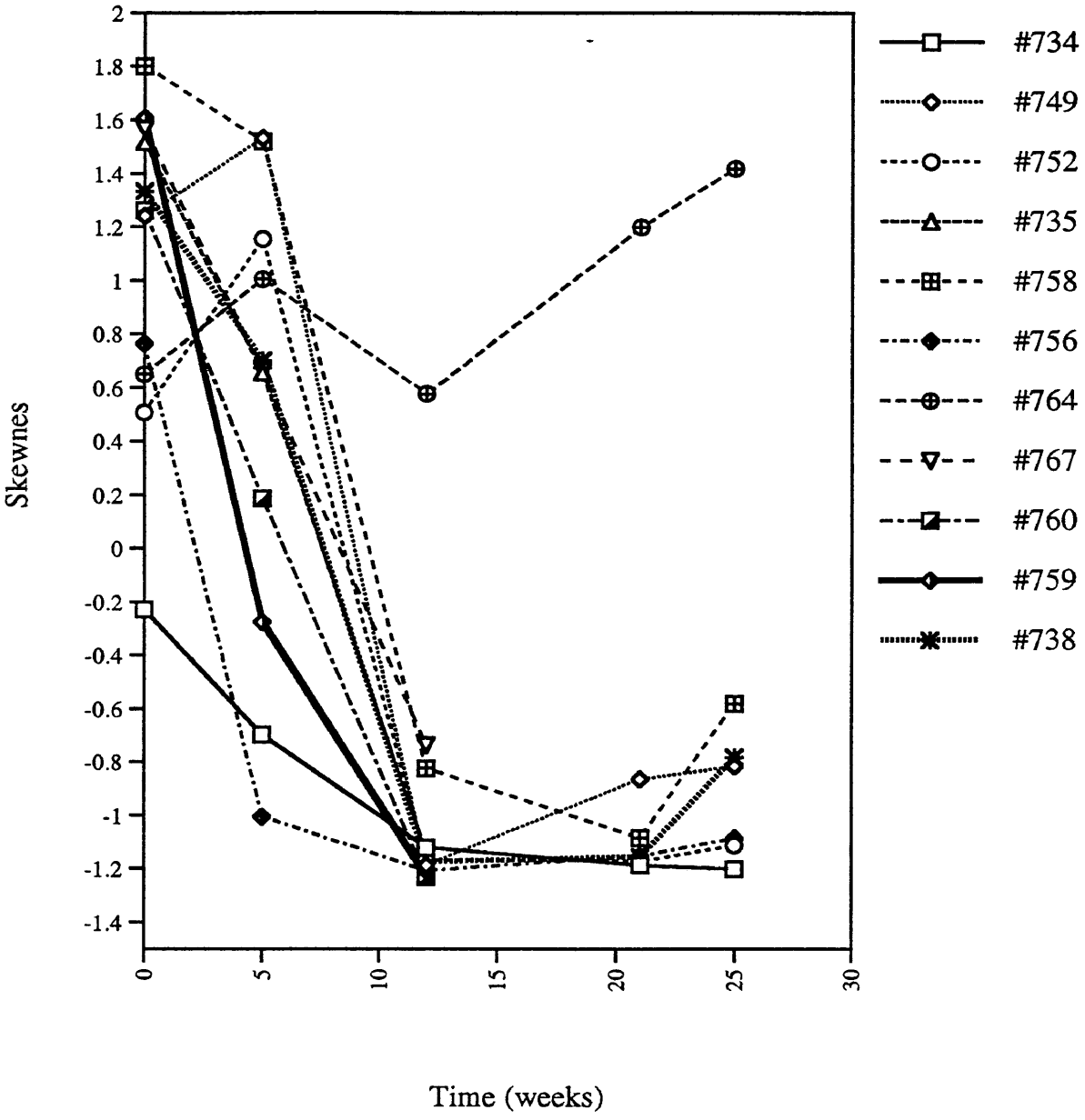


TABLE 6. Comparison of skewness indices between timepoints using a paired non-parametric paired sign test. Each paired sign test compared the skewness indices for the fish at each time point to the skewness indices of the same group of fish at the previous time point, considering the values for each individual at the two time points in pairs. The null hypothesis for each test states that there is an equal tendency for the skewness to be higher or lower than at the preceding time point. The p values were $>> 0.05$ in all cases except between week 5 and week 12, when the p value was 0.0010, indicating a significant shift in skewness between week 5 and week 12 but no significant change in skewness before week 5 and after week 12.

Comparison of skewness indices between time points by paired sign test

Time Points (paired)	p value
week 0, week 5	0.2266
week 5, week 12	0.0010*
week 12, week 21	0.6875
week 21, week 25	0.1250

*indicates a significant difference to a confidence of 0.05.

skewness index for an individual was just a likely to be higher than it was to be lower (i.e. there is no trend upwards or downward) at week 5 than at week 0, at week 12 than at week 5, at week 21 than at week 12 and at week 25 than at week 21. The results of the four paired sign tests are summarized in Table 6. The p values were 0.2266 for week 0 and week5, 0.0010 for week 5 and week 12, 0.6875 for week 12 and week 21 and 0.1250 for week 21 and week 25. It appears that there were no significant trends upward or downward with respect to the skewness index from week 0 to week 5, from week 12 to week 21 and from week 21 to week 25. However, there was a significant ($p = 0.0010$) tendency for a change in skewness index from week 5 to week 12. From Figure 21 it is clear that this is a downward trend. Therefore the skewness index decreased significantly (becomes more negative) from week 5 to week 12, but did not change significantly before week 5 or after week 12. Concurrent with this shift in skewness was the increase in average affinity of antibodies with time. These two observations imply that a shift occurred from a more positively skewed distribution with a predominance of higher affinity antibodies early in the immune response to a negatively skewed one with a predominance of higher affinity antibodies later in the immune response. The average affinity values for the eleventh individual (#764) were consistently low throughout, suggesting that this particular fish did not undergo affinity maturation nor did it experience a shift in favor of high affinity subpopulations.

In vitro antibody affinity distributions

A set of peripheral blood lymphocyte cultures was stimulated by a mitogen, *E. coli* lipopolysaccharide (LPS), which is a T cell-independent non-specific activator of B cells. LPS was used to stimulate all or most of the B cells present. Another set of cultures was incubated with the antigen trinitrophenylated-lipopolysaccharide (TNP-LPS) to stimulate TNP-specific B cells, also in a T-independent fashion. The T-independent carrier LPS was

selected to elucidate the proliferation of specific B cells without the added complication of T cell help.

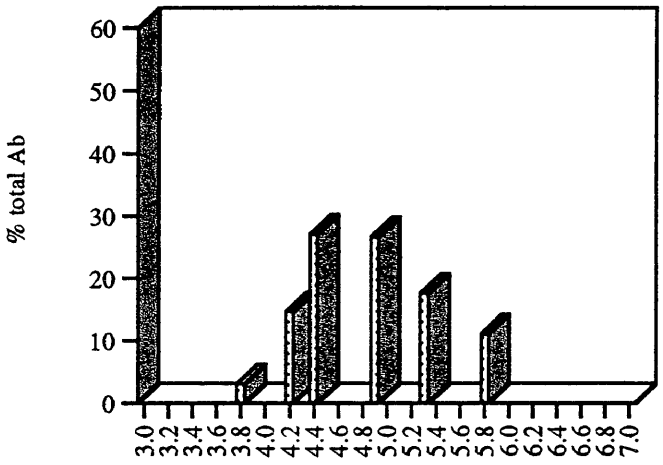
The culture supernatants obtained were analyzed for antibody concentrations and affinities. The supernatants from cultures set up at week 0 did not have detectable antibody titers using the methods employed in this study. At the other time points (weeks 5, 12, 21 and 25), some individuals did not have sufficient antibody in the culture supernatants to conduct an affinity ELISA. In all seven individuals for which affinity data was obtained, the supernatants at week 5 from LPS-stimulated cells displayed a similar affinity distribution as that of the corresponding plasma, i.e. the range of affinities and the relative percentages of antibody in each subpopulation were similar. Figures 24-26 show the affinity distributions of antibodies obtained *in vivo* and *in vitro* by LPS stimulation for several individuals. The supernatants from cells stimulated with 1 μg/ml TNP-LPS display an affinity distribution that is similar to that of corresponding LPS-stimulated culture supernatants. In contrast, the affinity distributions of antibodies from the 0.01 μg/ml TNP-LPS stimulated cultures had a greater proportion of high affinity antibodies than did antibodies from 1 μg/ml TNP-LPS stimulated cultures and LPS stimulated cultures (Figures 27-30).

The affinity distributions of antibodies from the various *in vitro* cultures were monitored over time. Comparing the affinity distributions of antibodies from LPS stimulated cultures at weeks 12, 21 and 25 (Figures 31-32), the percentage of antibodies in the lower affinity subpopulations (4.0 - 4.9) either remains constant or increases from week 12 to week 21 but decreases by week 25. As time progresses the percentage of antibodies in the higher affinity subpopulations (5.0 - 5.9) increases. It was also observed that the highest affinity antibody (6.0 - 6.2) was not observed at weeks 12 and 25 but was present in low amounts at week 21. When the affinity distributions of antibodies from cultures stimulated with 1 ug/ml TNP-LPS were compared over time, a similar pattern was observed (Figures 33-34). In the affinity distributions of 0.01 μg/ml TNP-LPS cultures

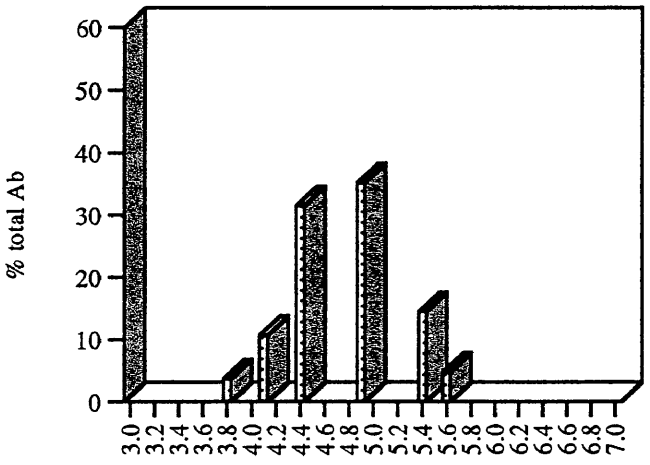
(Figure 35) , the percentage of antibodies in affinity subpopulations in the 4.0 - 5.0 range remained fairly constant from week 12 to week 25. In one individual there was an increase in the percentage of antibody in affinity subpopulations in the 5.2 - 5.4 range at week 25 compared to week 21. Once again antibody in the highest affinity subpopulations (6.1 - 6.2) was present only at week 21.

FIGURE 24. Comparison of affinity distributions of antibodies in plasma and from LPS stimulated cultures for fish #738.

Comparison of antibody affinity distributions of plasma and from LPS-stimulated cultures for fish #738 at week 12.



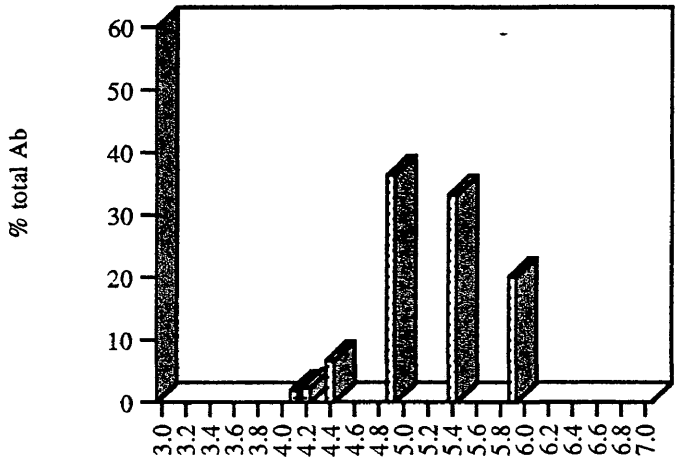
plasma



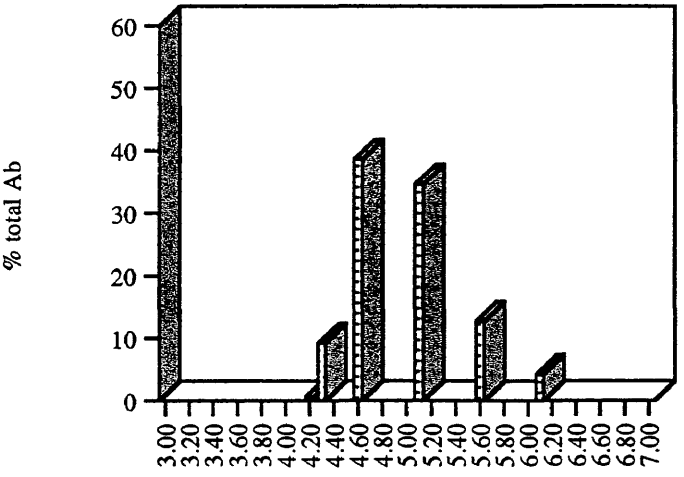
LPS

FIGURE 25. Comparison of affinity distributions of antibodies in plasma and from LPS stimulated cultures for fish #737.

Comparison of affinity distributions of antibody in plasma and from LPS stimulated cultures for fish #737 at week 21.



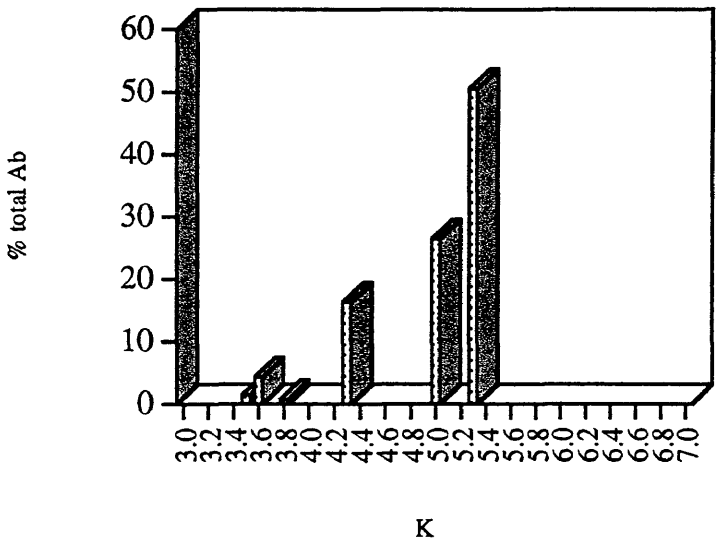
plasma



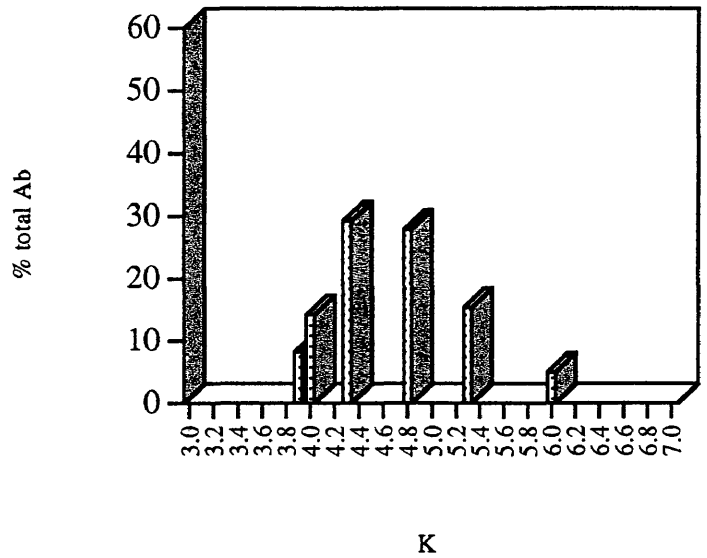
LPS

FIGURE 26. Comparison of affinity distributions of antibodies in plasma and from LPS stimulated cultures for fish #734.

Comparison of affinity distributions of antibodies in plasma and from LPS stimulated cultures for fish #734 at week 21.



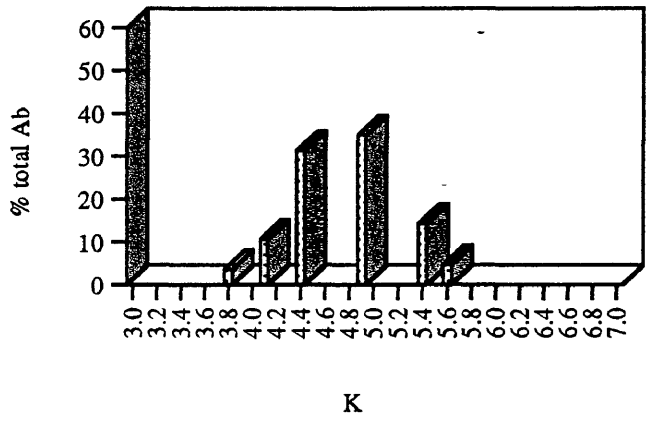
plasma



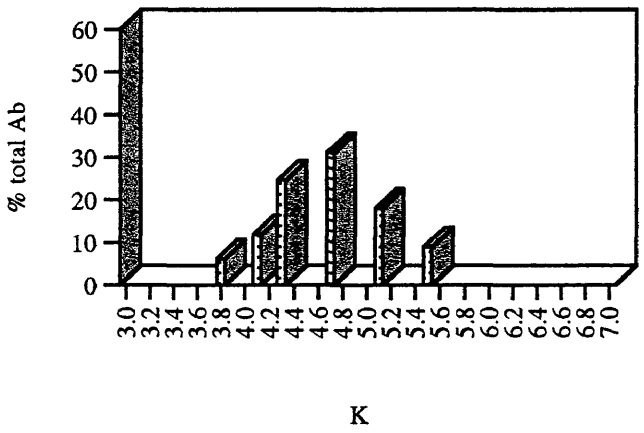
LPS

FIGURE 27. Comparison of affinity distributions of antibodies from cultures stimulated with LPS, 1 μg/ml TNP-LPS and 0.01 μg/ml TNP-LPS for fish #738.

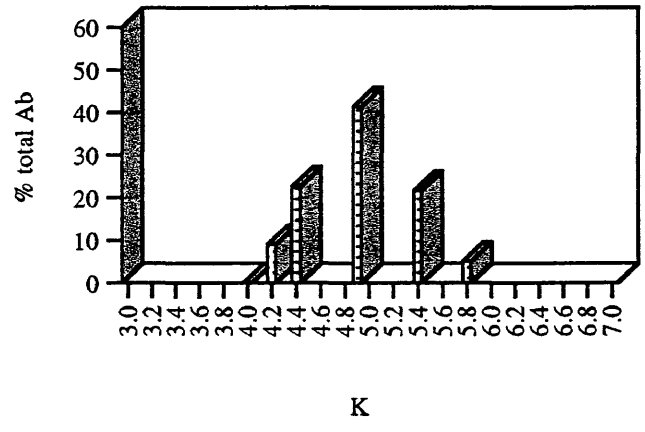
Comparison of affinity distributions of antibodies from cultures stimulated with LPS, TNP-LPS (1 μg/ml) and TNP-LPS (0.01 μg/ml) from fish #738 at week 12.



LPS



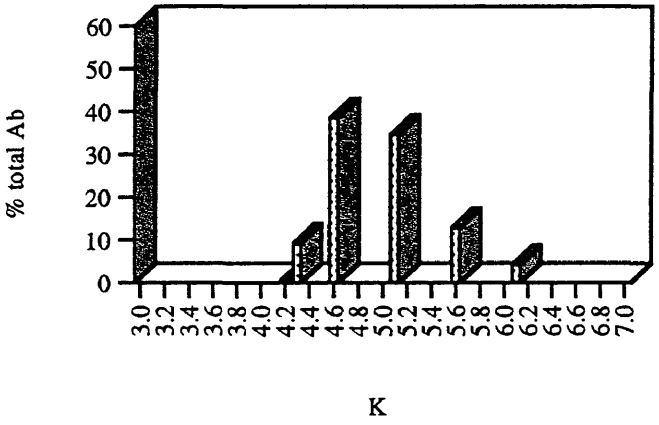
TNP-LPS (1 μg/ml)



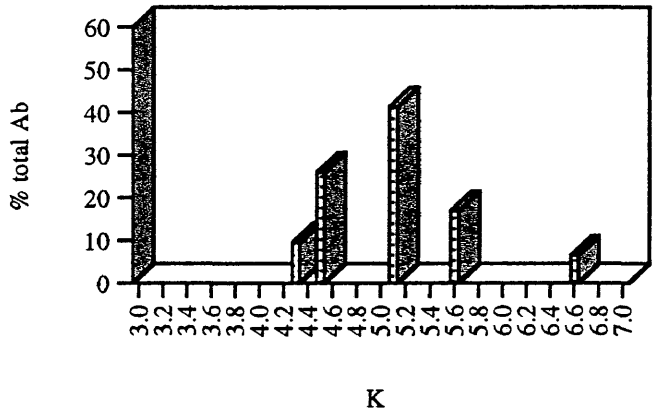
TNP-LPS (0.01 μg/ml)

FIGURE 28. Comparison of affinity distributions of antibodies from cultures stimulated with LPS, 1 μg/ml TNP-LPS and 0.01 μg/ml TNP-LPS for fish #737.

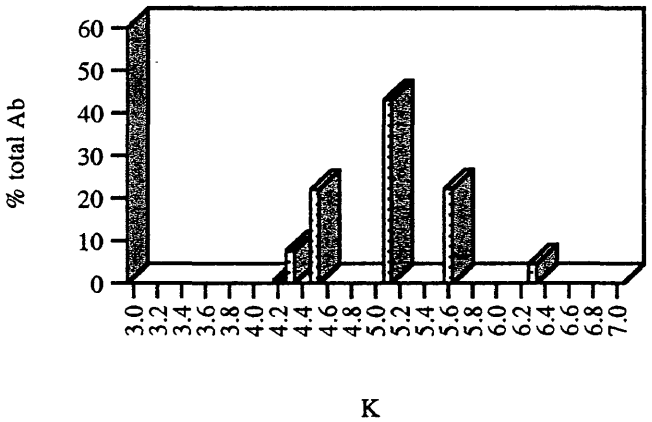
Comparison of affinity distributions for antibodies from cultures stimulated with LPS and 1 μg/ml of TNP-LPS and 0.01 μg/ml TNP-LPS for fish #737 at week 21.



LPS



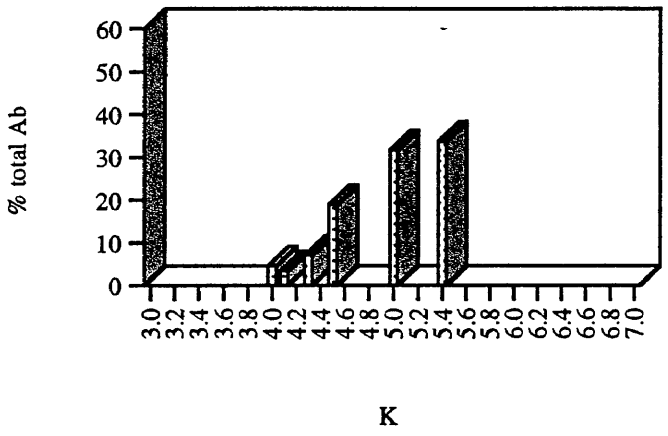
TNP-LPS (1 μg/ml)



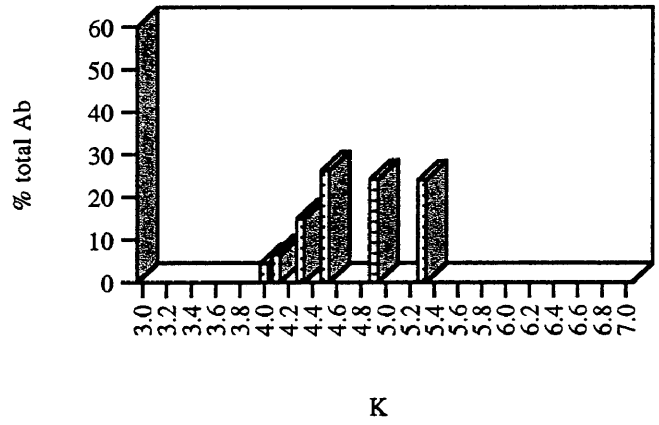
TNP-LPS (0.01 μg/ml)

FIGURE 29. Comparison of affinity distributions of antibodies from cultures stimulated with LPS, 1 μg/ml TNP-LPS and 0.01 μg/ml TNP-LPS for fish #734.

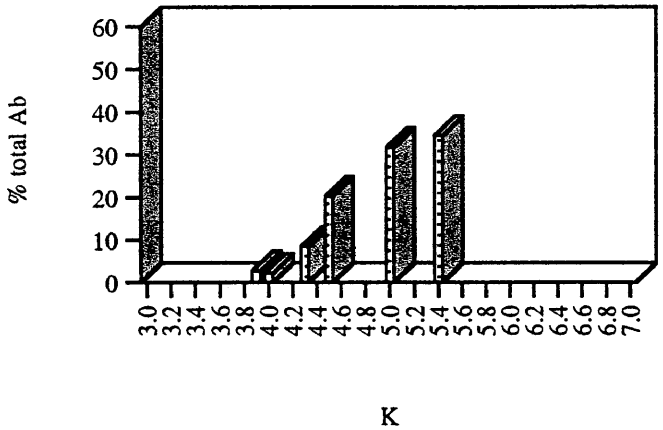
Comparison of affinity distributions of antibodies fromcultures stimulated by LPS, 1 μg/ml TNP-LPS and 0.01 μg/ml TNP-LPS for #734 at week 25.



LPS



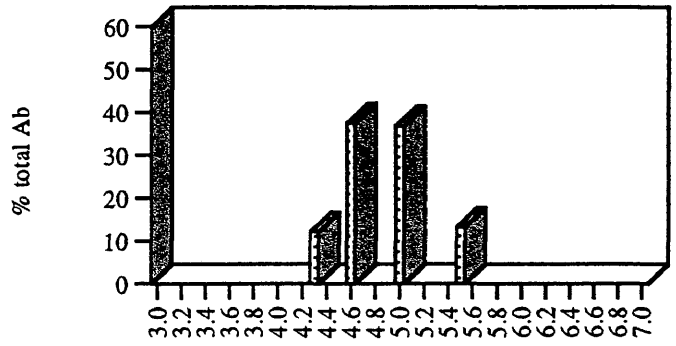
TNP-LPS (1 μg/ml)



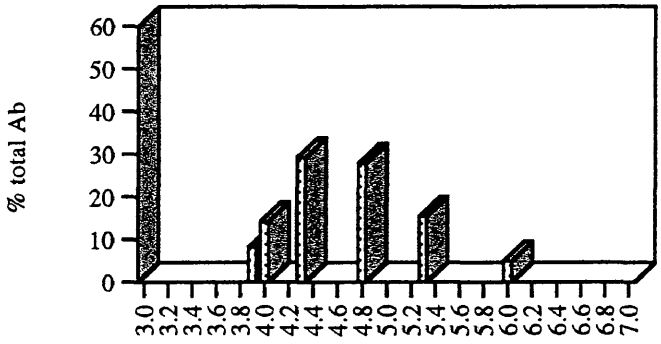
TNP-LPS (0.01 μg/ml)

FIGURE 30. Comparison of affinity distributions of antibodies from LPS stimulated cultures at weeks 12, 21 and 25 for fish #734.

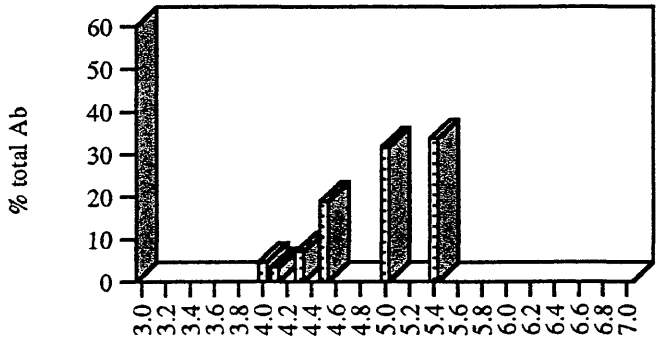
Comparison of affinity distributions of antibodies from LPS stimulated cultures at weeks 12, 21 and 25 for fish #734.



week 12



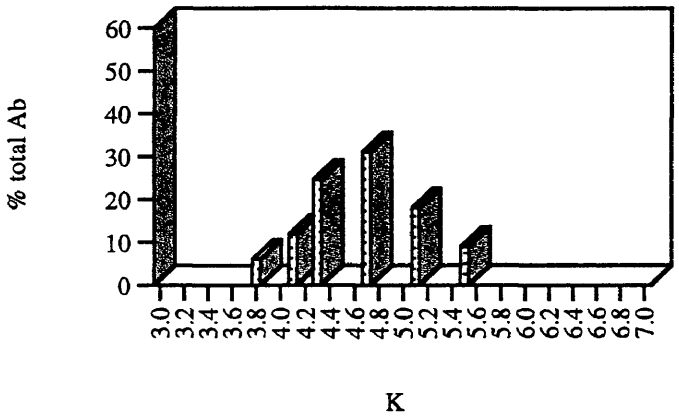
week 21



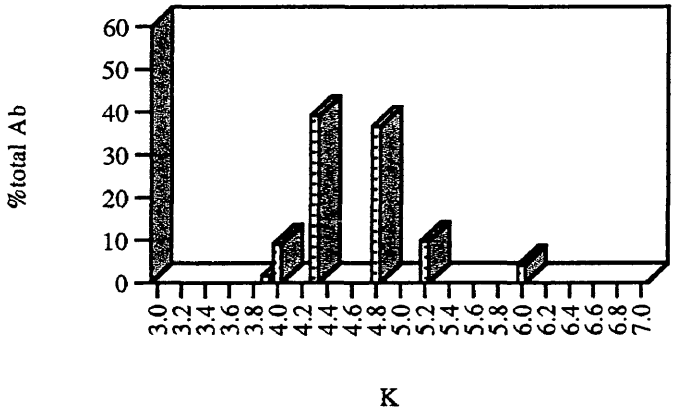
week 25

FIGURE 31. Comparison of affinity distributions of antibodies from LPS stimulated cultures at weeks 12 and 21 for fish #738.

Comparison of affinity distributions of antibodies from cultures stimulated with 1 μg/ml TNP-LPS at weeks 12 and 21 for fish #738.



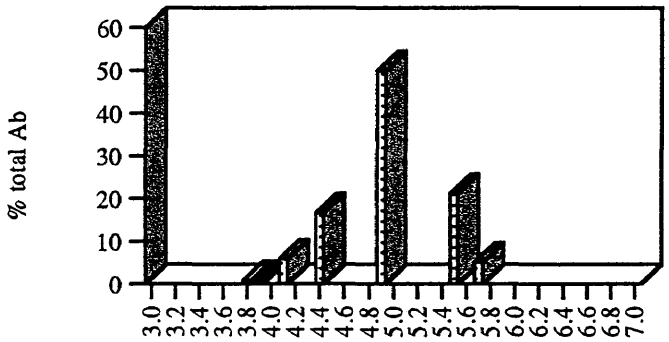
week 12



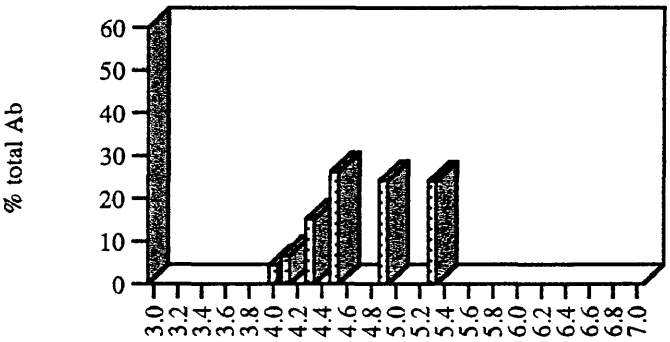
week 21

FIGURE 32. Comparison of affinity distributions of antibodies from cultures stimulated with 1 μg/ml TNP-LPS at weeks 12 and 25 for fish #734.

Comparison of affinity distributions of antibodies from cultures stimulated with 1 μg/ml TNP-LPS at weeks 12 and 25 for fish #734.



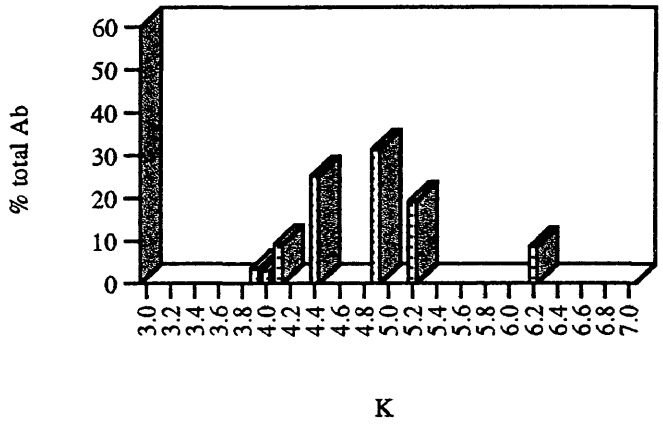
week 12



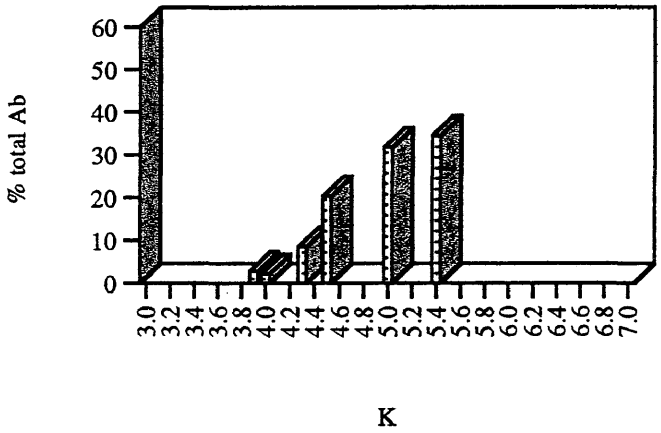
week 25

FIGURE 33. Comparison of affinity distributions of antibodies from cultures stimulated with 0.01 μg/ml TNP-LPS at weeks 21 and 25 for fish #734.

Comparison of affinity distributions of antibodies from cultures stimulated with 0.01 μg/ml TNP-LPS at weeks 21 and 25 for fish #734.



week 21



week 25

DISCUSSION

In mammals, the average intrinsic affinity of antibodies can increase by several logs during the immune response to an antigen. A smaller increase in antibody affinity has been observed in several teleost species, including the catfish (Lobb, 1985), Atlantic salmon (Killie et al., 1991), coho salmon (Voss et al., 1978) and carp (*Cyprinus carpio*) (Fiebig et al., 1977). In all cases the average intrinsic affinity did not increase by more than a log.

In the majority of rainbow trout (*Oncorhynchus mykiss*) examined in this study, the average affinity of secreted antibody increased from week 0 to week 21, that is, during the period of the primary immune response. There was a maximum of a log increase in average intrinsic affinity during this time period for the individuals studied. The average affinity then either remained constant or decreased from week 21 to week 25 after a second injection of antigen at week 21. It has been suggested that the relatively low increase in intrinsic affinity of teleost antibody may be compensated by the multivalent (multiple antigen-binding sites) nature of the teleost tetrameric Ig molecule, leading to a higher functional affinity or avidity (Voss et al., 1978). Fiebig et al. (1977) demonstrated that while the intrinsic affinity values for carp anti-dinitrophenyl (DNP) antibodies increased only slightly during the immune response, the functional affinity of the antibodies increased 100 fold. Thus the fish Ig molecule may not require the large increase in intrinsic affinity experienced by the monomeric mammalian IgG molecule in order to have a comparable functional affinity (Kaattari, 1994).

In this study the titers of the immunized rainbow trout increased with time from week 0 to week 21 and then either decreased or remained constant from week 21 to week 25. This can be explained by an antigen-driven selection mechanism. As the level of antigen declines with time fewer B cells are stimulated to proliferate, causing the rate of antibody produced to decrease. With a second, smaller injection of antigen the level of antibody starts to increase again. The pattern of affinity increase in the rainbow trout

antibodies is also consistent with the theory that antigen-driven clonal selection is the mechanism behind affinity maturation in fish. The clonal selection theory was described for mammals by Burnet (1959). One of the postulates of this theory is that prior to contact with antigen, each antibody-producing cell differentiates to produce a single antibody with a unique specificity. The clonal selection theory can be expanded to explain the change in antibody affinity with time following immunization. Antigen acts as a selecting factor by binding to B cells with antibody receptors specific for the antigen. The binding of antigen to these antibody receptors stimulates the B cells to proliferate and produce antibody identical to that of the receptors (with a carboxy secretory tail) (Goidl et al., 1968).

According to the theory, in the initial period following immunization, there is sufficient antigen to stimulate both low and high affinity B cells, leading to the production of antibodies with a range of affinities. As time progresses the level of antigen in the animal declines so that there is a preferential stimulation of high affinity B cells over low affinity cells since only high affinity B cells will be able to bind sufficient antigen to become activated. This leads to an increase in the proportion of high affinity antibodies relative to low affinity antibodies (Siskind and Benacerraf, 1969). By the same argument, early in the immune response there should be a greater proportion of low affinity antibodies than is present later since the greater amount of antigen allows more low affinity B cells to be stimulated. This is exactly what was observed in the antibody response of the immunized rainbow trout by analysis of the antibody subpopulations by an affinity ELISA method. However, in contradistinction to previous studies in most fish and mammals, within the range of affinities, there was a shift from predominantly lower affinity antibodies early in the primary immune response (weeks 0 and 5) to predominantly higher affinity antibodies late in the primary immune response (weeks 12 and 21) and in the secondary immune response (week 25). The decrease in average affinity in the secondary immune response can be explained by the fact that the B cells encounter a second bolus of antigen at week 21. This antigen bolus probably stimulated more low affinity B cells which

proliferated and produced low affinity antibodies, decreasing the average affinity of the plasma antibody. An alternative explanation is that, in those fish that exhibited a decrease after week 21, the high affinity B cells might have been selectively tolerized to the antigen, as has been suggested by Goidl et al. (1968) in the immune response of guinea pigs. The inhibition of high affinity B cells was not evident earlier in the immune response because at that time, the high affinity cells constituted a relatively small proportion of the total cell population. In a few animals the antibody affinity remained constant from the end of the primary response (week 21) to the secondary response (week 25). It is possible that most of the high affinity B cells had been activated by the first injection of antigen so that the affinity had already reached its maximum by the end of the primary response. The above observations are consistent with an antigen-driven selection mechanism, and we suggest that this mechanism underlies the development of affinity maturation in rainbow trout, and possibly in all teleosts. One individual (#764) did not show an increase in average affinity with time (Table 2). These results can be explained by the failure of this individual to establish an immune response as evidenced by the lack of increase in antibody titer following immunization (Table 1). It is not clear why this individul responded differently from the others, but as these were outbred animals the capability to respond to any one antigen can be variable.

Monitoring the skewness indices of the affinity distributions over time represents a novel method of characterizing affinity maturation, although Werblin at al. (1992) mentioned the skewness of rabbit affinities over time, no one as yet has published the evaluation of skewness indices for the study of affinity maturation. In a plot of skewness versus time, ten out of eleven individuals demonstrated a sharp decrease in skewness from week 5 to week 12. Considering the skewness to be random, it may be thought of as comparable to a situation where a coin is tossed eleven times. There is an equal probability (50%) at each toss that the coin will come out either heads or tails. If the coin is fair, the probability that it would come out say, heads, ten out of eleven times is extremely small

(1:2048) and such a result would indicate a loaded or biased coin. Similarly, at each time point the skewness value, if random, has an equal probability of being higher or lower than the value at the previous time point. With these odds, the probability of the result being the same (i.e. every time higher or every time lower) ten out of eleven times is extremely small. The fact that ten out of eleven individuals demonstrate progressively lower (more negative) values at each subsequent time point indicates that there is a considerable trend towards a more negative skewness with time. Therefore the distribution of the affinities is becoming more skewed toward higher affinities over time.

A paired non-parametric test, the paired sign test, was used to determine if there was a significant change in skewness with time. Each paired sign test compared the skewness indices of a group of fish at a particular time point with the skewness indices of the same group of fish at the previous time point, considering the skewness indices of each fish at two different time points in pairs. There were no significant changes in the skewness from week 0 to week 5, from week 12 to week 21 and from week 21 to week 25. However, there was a significant decrease in skewness towards a more negative value from week 5 to week 12 ($p = 0.0010$ in a paired sign comparison of indices at week 5 and week 12). Concurrent with the trend towards a more negative skewness was the increase in the average affinity of the antibodies. A correlation can be made between a positive skewness index, such as is observed at week 0 and week 5, and a predominance of low affinity antibodies (also observed at week 0 and week 5) and between a negative skewness and a predominance of high affinity antibodies. This would suggest that, as there is a significant trend from a more positive skewness to a much more negative skewness from week 5 to week 12, there is likewise a shift in predominance from low affinity to high affinity antibodies in this window of time. Before week 5 and after week 12, there does not appear to be significant shifts in predominance among the antibody affinity subpopulations. The above observations could be explained by an antigen-driven selection mechanism whereby the antigen is introduced into the body at week 0, after which its level

steadily declines with time. Somewhere between week 5 and week 12, the antigen reaches a concentration that is low enough to preferentially stimulate high affinity B cells over low affinity ones, leading to the observed shift in favor of high affinity antibodies. The antigen level might continue to decline from week 12 to week 21, but this lower concentration of antigen does not appear to stimulate a population of higher affinity B cells than was observed earlier in the response from week 5 to week 12. These results could provide an explanation for the lack of affinity maturation or low increases in affinity reported by previous researches (Lobb, 1985; Killie et al., 1991). These studies started monitoring the antibody affinities only after week 12. Thus the antibodies might already have undergone a maturation response and their affinities might have plateaued by the time the monitoring was started. Following secondary immunization with the same antigen, the second bolus of antigen simulates high affinity B cells that were already present prior to immunization. However, there do not appear to be considerably heightened titers or a significant shift toward higher affinity antibodies following secondary immunization.

In mammals, the diversity of antibodies available for antigen selection is generated by recombination of the immunoglobulin (Ig) gene segments and somatic hypermutation of the immunoglobulin gene (Tonegawa, 1983; Griffiths et al., 1984). All vertebrates that have been examined, including the primitive shark *Heterodontus* (Hinds-Frey et al., 1993), *Xenopus* (Wilson et al., 1992), chicken (Reynaud et al., 1989), rabbit, sheep and man, demonstrate somatic hypermutation of Ig genes. The observed mutation rate is one point mutation in 1000 bp per cell generation, which is much higher than the spontaneous mutation rate. It is possible that the transcription initiation process and transcription-coupled repair (Neuberger and Milstein, 1987; Storb, 1996) might play a role in the generation of somatic hypermutation in Ig genes. The link between hypermutation and antibody affinity maturation has been demonstrated in mammals. Somatic hypermutation does not necessarily lead to an increase in the affinity of expressed antibody (Chen et al., 1995). However, when somatic mutations lead to the production of antibody of higher

affinity, the antigen selects these higher affinity B cells so that over time, the high affinity cells become enriched in the cell population (Neuberger and Milstein, 1987). Hypermutation-induced amino acid substitutions were shown to increase as antibody affinity matured in mice (Griffiths et al., 1984; Allen et al., 1988; Berek and Milstein, 1987) and humans (Bye et al., 1992).

Thus far there has been no published research on the generation of somatic hypermutation in teleosts. However, the organization of the teleost Ig heavy chain gene has been revealed to be similar to that of mammals (Amemiya and Litman, 1990; Ghaffari and Lobb, 1990; Roman and Charlemagne, 1994). The results of this project, in which antibody affinity was analyzed by partitioning a mixture of antibodies into affinity-based subpopulations, indicate an upward shift in affinity occurring in a teleost fish, the rainbow trout. This increase in average affinity with time suggests a possible somatic mutation mechanism that may provide new higher affinity antibodies for antigen-driven selection. The occurrence of somatic hypermutation in teleosts and possible underlying mechanisms is thus a prime target for future research.

The change in average affinity can be due either to the appearance of new antibody species or to a shift in the relative proportions of the different antibody species present in the total population (Siskind and Benacerraf, 1969). The possibility that the observed increase in affinity of the rainbow trout antibodies was due to the selection by antigen of new high affinity antibody variants was considered. The affinity distributions at the various time points appear to have defined upper and lower limits as there were no antibodies with affinities (K) lower than 3.8 and higher than 6.6. There were no new antibody subpopulations appearing later in the immune response that were not already present at the start of the experiment, which suggests that a shift in predominance occurred among various antibody forms that were already present in the fish at week 0. This shift, and not the appearance of new antibody variants, is sufficient to explain the observed increase in affinity. Comparing the affinity distribution profiles from week 0 to week 25,

there was no change in the range of affinities expressed. No new high affinity antibody subpopulations were observed over time that might have been responsible for the increase in affinity. There are several alternative mechanisms that could explain the lack of new antibody variants; 1) no somatic mutations occurred, 2) somatic mutations occurred but they did not generate high affinity antibody binding sites and 3) somatic mutations occurred but the high affinity variants were not selected and possibly even down-regulated. Addtional research using molecular techniques to examine somatic mutation in Ig genes before and after contact with antigen is required to elucidate the actual operative mechanism.

In vitro stimulation of peripheral blood lymphocytes from the blood samples taken at weeks 0, 5, 12, 21 and 25 were used to evaluate the cellular mechanism of *in vivo* affinity maturation as well as the capability of the cells to undergo antigen-driven selection of the high affinity B cells. Polyclonal activation of peripheral blood lymphocytes at the various time points produced in vitro antibodies with affinity distributions paralleling that observed in the plasma. This finding substantiates the idea that the process of affinity maturation *in vivo* must rely strongly on preferential clonal expansion of high affinity, antigen-specific B cells. A similar shift was observed in supernatants from cultures stimulated with lipopolysaccharide (LPS) and trinitrophenylated lipopolysaccharide (TNP-LPS). There was a shift from a greater predominance of low affinity antibodies to a greater predominance of higher affinity antibodies over time in the LPS profiles, indicating that an enrichment of high affinity cells occurred as time progressed since the mitogen LPS non-specifically stimulates all the B cells that are present. The low affinity subpopulations were present even late in the immune response, albeit at a lower proportion than the high affinity subpopulations. It is probable that there is an upper limit to the affinity of the B cells in the animal, as determined by such factors as somatic mutation and combinatorial joining in the Ig genes. There also appears to be an upper limit on the degree of restriction to high affinity B cells. Both *in vivo* and *in vitro*, a shift in predominance towards a greater

proportion of high affinity B cells was observed with time but although the proportion of low affinity B cells declined temporally, they were still present late in the primary response and in the secondary response. It is possible that these low affinity B cell subpopulations may possess a better potential specificity for some other antigen and are thus retained for this purpose.

The effect of different doses of antigen, TNP-LPS, on the B cell population was elucidated by using a high dose (1 μg/ml) and a low dose (0.01 μg/ml) of antigen in *in vitro* cultures. At weeks 5, 12, 21 and 25, the affinity distributions indicated a greater predominance of higher affinity antibodies with the low dose than with the high dose of antigen. This indicates that the low dose of antigen preferentially stimulates high affinity B cells to proliferate, leading to the observed shift towards higher affinity antibodies. This provides further support for an antigen-driven clonal selection mechanism underlying the affinity maturation process in rainbow trout. When antigen is present in limiting amounts, it preferentially binds high affinity B cell receptors, leading to their subsequent proliferation and predominance in the B cell population.

Besides contributing to the limited body of work on affinity maturation in teleosts, these findings have important applications on the vaccination and general health care of fish. The purpose of vaccination is to induce the protective immune response of an animal against disease agents. Since antibodies with a greater affinity for a particular antigen are more efficient at effecting their removal from the animal, effective vaccination and immunization should attempt to induce these high affinity antibodies. The results give an idea of the timeframe in which high affinity antibodies are induced and possibly when the best protection may be attained.

SUMMARY

The main findings and conclusions of this project can be summarized in several points:

1. Utilizing a solid-phase affinity ELISA that partitions antibodies into subpopulations based on their affinity, a shift in predominance from low affinity antibodies to high affinity antibodies over time was observed in rainbow trout immunized with the antigen TNP-KLH. This shift was characterized by monitoring the skewness indices of the affinity distributions of antibodies at various time points. The skewness became considerably more negative from week 5 to week 12 but did not change significantly before week 5 and after week 12.

2. Based on the observed increase in average affinities with time, an antigen-driven clonal selection mechanism for affinity maturation was postulated. The results from the *in vitro* studies suggest that antigen-driven selection is the mechanism underlying affinity maturation in rainbow trout, similar to the situation in mammals. Combining these findings with those in 1 above, it is proposed that antigen becomes limiting between week 5 and week 12 of the immune response and preferentially selects high affinity B cells for proliferation, leading to the observed upward shift in affinity between week 5 and week 12. The pinpointing of this window of time when antigen selection leads to affinity maturation could explain why previous studies of affinity maturation in teleosts found little to no increase in antibody affinity, since these studies started monitoring affinity only after week 12.

3. The carcinogen aflatoxin B_1 (AFB_1) did not appear to have a significant effect on the rainbow trout antibody titer and average affinity throughout the immune response when fish were embryologically exposed to it.

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